Ceruloplasmin is a copper-binding protein, which is the major ferroxidase in plasma of hepatic origin. We now provide evidence for a novel membrane-bound form of ceruloplasmin expressed by astrocytes in the mammalian central nervous system. Using a monoclonal antibody (1A1), we show that the cell surface antigen recognized by this antibody is ceruloplasmin and that it is directly anchored to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. Our peptide mapping and other immunochromesical studies indicate that, except for the GPI anchor, the membrane-bound and secreted plasma forms are similar. We also show that the membrane-bound form of ceruloplasmin has oxidase activity. These studies therefore suggest that the GPI-anchored form of ceruloplasmin may play a role similar to the secreted form in oxidizing ferrous iron. The GPI-anchored form of ceruloplasmin expressed by astrocytes is likely to be the major form of this molecule in the central nervous system because serum ceruloplasmin does not cross the blood-brain barrier. Lack of this form of ceruloplasmin in the central nervous system could lead to the generation of highly toxic free radicals, which can cause neuronal degeneration as seen in aceruloplasminemia and other neurodegenerative diseases such as Parkinson’s and Alzheimer’s disease.

Iron plays an important role as a cofactor for various enzymes, such as the cytochromes of the electron transport chain and ribonucleotide reductase. On the other hand, free iron can generate highly toxic free radicals because it is a redox-active transition metal (1). A number of enzymes, binding proteins, and transporters have been identified that are involved in mobilizing, transporting, and sequestering iron (1–3). Recent studies on the yeast Saccharomyces cerevisiae have resulted in the identification of several proteins, such as Fet3 and Ftr1, which directly participate in iron transport in this organism (4–6). The mammalian homologues of many of these proteins have yet to be identified. Ceruloplasmin, the major ferroxidase of plasma (300–450 μg/ml), is required for iron transport by transferrin. The oxidation of ferrous iron (Fe(II)) to ferric iron (Fe(III)) mediated by ceruloplasmin is necessary for iron incorporation into transferrin, since transferrin only binds the ferric form of iron. As a ferroxidase, ceruloplasmin might also play a role in a transferrin-independent iron uptake system, such as the one identified by Kaplan and colleagues (7), which requires reduction of iron at the cell surface (reviewed in Ref. 1).

Direct evidence for the role of ceruloplasmin in iron metabolism comes from studies of individuals with aceruloplasminemia, a hereditary deficiency of ceruloplasmin (8–15). These individuals have very little or undetectable levels of ceruloplasmin and severe intracellular iron accumulation in a number of organs, including the brain, particularly in the deep extrapyramidal motor nuclei, where it is associated with neurodegeneration. The neurodegeneration is likely to be a consequence of oxidative stress induced by the oxidation of ferrous iron by agents such as hydrogen peroxide (1). In support of this, Miyajima et al. (16) reported a dramatic increase in the levels of lipid peroxidation in the plasma of individuals with aceruloplasminemia. A number of other neurodegenerative diseases, including Parkinson’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis, multiple sclerosis, and Hallervorden-Spatz disease are also associated with altered brain iron metabolism and free radical injury (17). It is therefore possible that ceruloplasmin might contribute to the pathology seen in these neurodegenerative diseases as well.

Although generally considered a soluble plasma protein of hepatic origin, we now provide evidence using a monoclonal antibody, mAb 1A1, of a novel GPI-anchored form of ceruloplasmin that is localized to the surface of astrocytes in the central nervous system. The cell surface localization of ceruloplasmin is not seen on hepatocytes and cells of the choroid plexus, both of which are known to secrete ceruloplasmin. Since iron deposition occurs in the brain in aceruloplasminemia and because the level of the secreted form of ceruloplasmin in the cerebrospinal fluid is very low, this novel membrane-associated form of ceruloplasmin is likely to play an important role in iron metabolism in the central nervous system.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Astrocyte cultures were prepared from neonatal rat cerebral cortex as described previously (18). Astrocytes and C6 glioma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, vitamins, and penicillin and streptomycin (Life Technologies, Inc.) in tissue culture flasks (Nunc). For certain immunocytchemistry experiments and the ferroxidase assay, cells were replated in serum-free Neurobasal Medium with G-5 supplement (Life Technologies, Inc.).

**Preparation of Membrane Protein Extract—**Membrane protein extracts were prepared from C6 glioma cell tumors raised in nude rats. Tumors were generated by injecting approximately 5 × 10^6 cultured C6 cells intracranially into athymic nude rats. The tumors were collected and homogenized in a glass homogenizer using a UP-500 Ultraturrax, 60 Hz, 500 rpm (Janke & Kunkel, GmbH) in ice-cold Tris-buffered saline solution; GFAP, glial fibrillary acidic protein.
glioma cells subcutaneously into Nu/Nu nude rats (Charles River). After 1–2 weeks, tumors were harvested and homogenized using a motor-driven Dounce homogenizer in ice-cold hypertonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 5 mM KCl, pH 7.4) containing phenylmethylsulfonyl fluoride and soybean trypsin inhibitor (both at 50 μg/ml), leupeptin (5 μg/ml) and pepstatin (both at 5 μg/ml), and NaN₃ (0.02%). The homogenate was first centrifuged at 1000 × g for 10 min, and the resulting supernatant centrifuged at 100,000 × g for 60 min. The pellet was then solubilized in ice-cold 1% Nonidet P-40 solubilization buffer (150 mM NaCl, 25 mM Tris, 0.2 mM MgCl₂, 0.2 mM CaCl₂, pH 7.4 (TBS), 1% Nonidet P-40, 0.02% NaN₃) containing protease inhibitors (as above), and the supernatant clarified by centrifugation at 100,000 × g for 30 min. Detergent extracts of purified membranes of cultured C6 glioma cells were also made using a similar protocol.

Purification of the 1A1 Antigen—An immunoaffinity column consisting of mAb 1A1 conjugated to cross-linked agarose beads (Affi-Gel-10; Bio-Rad) was made according to the manufacturer’s instructions. Briefly, 2 ml of Affi-Gel-10 beads was added to 5 ml of purified mAb 1A1 in phosphate-buffered saline (3 mg/ml) and incubated for 4 h at 4 °C. After washing with phosphate-buffered saline, the beads were blocked with 0.5% ethanolamine (pH 7.4) for 30 min. A 5-ml column of Affi-Gel-10 beads conjugated to ovalbumin (10 mg/ml beads) was also made using the same protocol.

The 135-kDa band was excised for sequencing. Twenty of the positions gave weak signals (italics). A novel GPI-anchored Ceruloplasmin

RESULTS

Purification and N-terminal Amino Acid Microsequencing of the 1A1 Antigen—Immunoaffinity chromatography was used to purify the 1A1 antigen in sufficient quantity for N-terminal amino acid microsequencing. We have previously shown that only astrocytes express this antigen in the central nervous system (18). Because of the difficulty in using astrocyte cultures for large scale purification, we sought an astrocytic cell line that expressed this molecule. C6 glioma cells were found to express the 1A1 antigen, both by immunocytochemistry and by immunoprecipitation after [35S]methionine labeling (18). These cells were therefore used for the purification of the molecule, since they can be grown easily in culture or be injected subcutaneously into nude rats to generate tumors that yield large amounts of material. Nonidet P-40 extracts of a membrane preparation from either C6 glioma tumors or cultured C6 glioma cells were used to purify the 1A1 antigen. Fractions eluted from the mAb 1A1 immunoaffinity column, and analyzed by SDS-PAGE and silver staining, showed a single band, of approximately 135 kDa (Fig. 1A). This molecular mass is similar to that reported previously for the 1A1 antigen immunoprecipitated from rat cortical astrocytes (18). Two-dimensional gel electrophoresis of the immunoaffinity-purified protein revealed the presence of only one polypeptide species (data not shown).

Partial N-terminal amino acid sequence data of the 1A1 molecule immunoaffinity-purified from C6 glioma tumors and electrophoresed onto a PVDF membrane yielded a 13-amino acid sequence. Two of the positions gave weak signals (italics). A search of protein data bases showed that this sequence (RE-
KHYYGITEAV) was identical to that of rat ceruloplasmin, a copper-binding protein of the same molecular weight as the 1A1 molecule (27, 28).

**1A1 Molecule Is Recognized by Anti-ceruloplasmin Antibody**—To further confirm that the immunoaffinity-purified protein was ceruloplasmin, the ability of a polyclonal anti-ceruloplasmin antibody to recognize this protein was determined. To this end, the immunoaffinity-purified 1A1 protein was Western blotted with a goat anti-human ceruloplasmin antibody. These Western blots demonstrated that the protein, purified from either C6 glioma tumors or cultured C6 glioma cells (Fig. 1B), is recognized by the anti-ceruloplasmin antibody. These results therefore provide additional evidence that the immunoaffinity-purified 1A1 protein is likely to be identical or highly similar to ceruloplasmin.

Since ceruloplasmin is one of the major protein components in serum (300–450 μg/ml), immunoprecipitations from serum with the mAb 1A1 were done to establish whether this antibody could recognize serum ceruloplasmin. The polyclonal anti-ceruloplasmin antibody could immunoprecipitate a 135-kDa molecule from rat and bovine serum. However, the mAb 1A1 precipitated ceruloplasmin from rat serum but not from bovine serum (data not shown).

**Ceruloplasmin Immunocytochemistry**—Immunofluorescence labeling with the mAb 1A1 showed labeling of ceruloplasmin on the surface of cultured neonatal rat astrocytes (Fig. 2, E and F), but not on cultured rat choroidal cells (Fig. 2, G and H) or hepatocytes (data not shown). To further confirm the cell surface ceruloplasmin labeling, astrocytes and C6 glioma cells were labeled with a polyclonal goat anti-ceruloplasmin antibody. These experiments also showed that ceruloplasmin was localized to the surface of these cells (Fig. 2, A–D). Since this polyclonal anti-ceruloplasmin antibody could recognize ceruloplasmin present in fetal bovine serum, the astrocytes and C6 glioma cells were grown in serum-free defined medium. Our earlier work has shown that the mAb 1A1 does not label other cells, i.e. neurons, oligodendrocytes, or endothelial cells, in the central nervous system (18).

**Ceruloplasmin Is Directly GPI-anchored to the Cell Surface**—

The immunofluorescence staining indicates that ceruloplasmin is present on the surface of astrocytes and C6 glioma cells. The cell surface localization of ceruloplasmin on astrocytes and C6 glioma cells is not likely to be the result of ceruloplasmin spanning the cell membrane, as it does not have a sufficiently long hydrophobic amino acid stretch that could serve as a membrane-spanning domain. It is therefore likely that the surface localization of ceruloplasmin is due to (i) association of ceruloplasmin with the extracellular matrix, (ii) association with a cell surface receptor, or (iii) a covalent attachment to the cell membrane. Because a sequence near the C-terminal region of ceruloplasmin meets the minimal requirement for a GPI anchor addition (29–31), we examined whether the cell surface localization of ceruloplasmin could be removed by treatment with PI-PLC, which specifically cleaves GPI anchors from GPI-anchored proteins. Astrocytes purified from the neonatal rat cerebral cortex and C6 glioma cells were treated with PI-PLC and stained with mAb 1A1. Incubation with this enzyme eliminated cell surface labeling by mAb 1A1 (Fig. 3), indicating that the localization of ceruloplasmin on the surface of astrocytes and C6 glioma cells is via a GPI-anchor. There are two possibilities regarding the association of ceruloplasmin to the surface of C6 glioma cells by a GPI anchor. (i) Ceruloplasmin might be associated with the cell surface via a receptor which is GPI-anchored to the membrane, or (ii) ceruloplasmin could be directly GPI-anchored to the cell surface. To resolve this issue, PI-PLC-released ceruloplasmin from C6 glioma cells was purified by mAb 1A1 immunoaffinity chromatography and then Western blotted with an antibody that specifically recognizes GPI anchors. This antibody strongly reacted with the ceruloplasmin purified from C6 glioma cells, but did not react with ceruloplasmin purified from rat serum (Fig. 4). Two-dimensional gel electrophoresis of the protein used for Western blotting revealed the presence of only one protein at 135 kDa, indicating that the anti-GPI antibody is not reacting to a protein copurifying with ceruloplasmin (data not shown). These results, therefore, suggest that ceruloplasmin on the surface of astrocytes and C6 glioma cells is directly GPI-anchored to the membrane.

**Cleveland Mapping of Ceruloplasmin**—We carried out peptide (Cleveland) mapping to determine if major differences in amino acid sequence exists between the GPI-anchored form and the secreted form of ceruloplasmin. Ceruloplasmin, immunoaffinity-purified (using the mAb 1A1) from either C6 glioma cell membranes or rat serum, was subjected to Staphylococcus V8 protease digestion. This treatment produced similar peptide maps of the GPI-anchored form and the soluble form of the molecule (Fig. 5), indicating that these two forms of ceruloplasmin are highly homologous.

**GPI-anchored Ceruloplasmin Has Oxidase Activity**—To assess whether the GPI-anchored form of ceruloplasmin has functional oxidase activity like the secreted form, the following experiment was carried out with C6 glioma cells. These cells were used since large numbers of cells were required (i.e. 90 confluent 75-cm² flasks). The cells were harvested and treated with PI-PLC to remove GPI-anchored molecules from the cell surface. The supernatant containing the latter was divided into two. One of these samples was immunodepleted of GPI-anchored ceruloplasmin with the mAb 1A1. The concentrated supernatants were separated by SDS-PAGE under nondenaturing conditions and the gels stained with p-phenylenediamine, which when oxidized produces a purple precipitate. A single 135-kDa band was observed in the PI-PLC-treated sample (Fig. 6). This band was lost in samples that were immunodepleted with the mAb 1A1 (Fig. 6). Nor was this band seen in control samples not treated with PI-PLC. These experiments, therefore, demonstrate that the GPI-anchored form of ceruloplasmin has oxidase activity. Since other bands were not visible, these studies also suggest that ceruloplasmin may be the major GPI-anchored oxidase on these cells.
DISCUSSION

We provide evidence that the 135-kDa cell surface molecule recognized by the mAb 1A1, which is expressed exclusively by astrocytes in the rat central nervous system (18, 32), is a novel GPI-anchored form of ceruloplasmin. The molecule recognized by the mAb 1A1 was purified by immunoaffinity chromatography using detergent-solubilized membrane extracts of C6 glioma tumors or cultured C6 glioma cells that also express this molecule. N-terminal microsequence analysis of the immunoaffinity-purified 135-kDa band indicated that the molecule recognized by the mAb 1A1 is identical (or homologous) to ceruloplasmin, which is classically considered a plasma protein of hepatic origin. Two-dimensional gel electrophoresis revealed the presence of only one 135-kDa polypeptide in the immunoaffinity-purified material, suggesting that mAb 1A1 recognizes only one molecule, namely ceruloplasmin. Additional evidence that the molecule recognized by this monoclonal antibody is ceruloplasmin (or homologous to it) was provided by the following experiments. (i) Western blot analysis demonstrated that the mAb 1A1-purified protein is recognized by a polyclonal anti-ceruloplasmin antibody, (ii) the monoclonal antibody 1A1 immunoprecipitates a 135-kDa protein from rat serum, and (iii) no differences were observed in the peptide fragments generated by Cleveland mapping of ceruloplasmin from serum and that from membrane preparations of C6 glioma cells.

We have also shown previously by metabolic labeling with \[^{35}\text{S}\text{]methionine}\ that this 135-kDa molecule is synthesized by astrocytes (18). Immunofluorescence labeling of cells \textit{in vitro} and iodination of cell surface proteins followed by immunoprecipitation showed that this molecule is associated with the plasma membrane (18). In addition, as shown for ceruloplasmin synthesized by liver cells \textit{in vitro} (28), we have shown that there is only a small reduction in the molecular weight of this molecule when astrocyte cultures are treated with tunicamycin (18), suggesting that it is poorly glycosylated. We have also reported previously that the 1A1 antigen, which we have shown here to be ceruloplasmin, increases in the cerebellum with postnatal development (32). Several earlier studies have reported the presence of ceruloplasmin mRNA in the brain (33–36). More recently, Klomp et al. (37) have reported ceruloplasmin gene expression by astrocytes. We now provide evidence of a novel GPI-anchored form of ceruloplasmin expressed on the surface of astrocytes in the mammalian central nervous system and that it has oxidase activity.

The cell surface localization of ceruloplasmin is unique to astrocytes, since cells of the choroid plexus and hepatocytes, both of which secrete ceruloplasmin, do not show surface labeling with mAb 1A1. Fibroblasts that form the fibroblastic capsule of various organs are the only other cell type to express this molecule on the cell surface (18). This cell surface localization of ceruloplasmin cannot be the result of ceruloplasmin spanning the cell membrane, since it does not have a suffi-
GPI-anchored ceruloplasmin has oxidase activity. GPI-anchored proteins, released from the cell surface of C6 glioma cells by PI-PLC treatment, were subjected to SDS-PAGE, and the gel was stained with p-phenylenediamine to reveal proteins with oxidase activity. The control sample, not treated with PI-PLC, does not contain any oxidase activity (lane 1). The PI-PLC-treated sample shows a 135-kDa band with oxidase activity (lane 2). This band is lost when the PI-PLC-treated sample is immunodepleted of ceruloplasmin with the monoclonal antibody mAb 1A1 (lane 3). Molecular size markers from the top are 250 and 98 kDa.

FIG. 6. GPI-anchored ceruloplasmin has oxidase activity. GPI-anchored proteins, released from the cell surface of C6 glioma cells by PI-PLC treatment, were subjected to SDS-PAGE, and the gel was stained with p-phenylenediamine to reveal proteins with oxidase activity. The control sample, not treated with PI-PLC, does not contain any oxidase activity (lane 1). The PI-PLC-treated sample shows a 135-kDa band with oxidase activity (lane 2). This band is lost when the PI-PLC-treated sample is immunodepleted of ceruloplasmin with the monoclonal antibody mAb 1A1 (lane 3). Molecular size markers from the top are 250 and 98 kDa.

FIG. 5. Peptide (Cleveland) mapping of GPI-anchored ceruloplasmin from C6 glioma cells and serum ceruloplasmin. GPI-anchored ceruloplasmin immunoaffinity-purified from cultured C6 glioma cells (lane 2) and immunoaffinity-purified rat serum ceruloplasmin (lane 3), yielded similar peptide maps when digested with S. aureus V8 protease and analyzed by SDS-PAGE and silver staining. Lane 1, protease alone. Lane 4, purified serum ceruloplasmin without enzyme treatment.

Novel GPI-anchored Ceruloplasmin

FIG. 4. Ceruloplasmin is GPI-anchored on C6 glioma cells. Western blotting for the GPI anchor is shown. GPI-anchored proteins were cleaved from the surface of C6 glioma cells by PI-PLC treatment. Ceruloplasmin was then purified by mAb 1A1 immunoaffinity chromatography and Western blotted using an antibody that specifically recognizes PI-PLC-released GPI-anchored proteins (anti-CRD; Oxford Glycosystems). The antibody strongly recognized ceruloplasmin from the C6 glioma cells (lane 1), but did not recognize ceruloplasmin purified from serum (5-fold excess; lane 2). Control blot in lane 3. Molecular size markers from the top are 200 and 116 kDa.

Protease and analyzed by SDS-PAGE and silver staining. Lane 1 (lane 2 from serum (5-fold excess; C6 glioma cells (cosystems). The antibody strongly recognized ceruloplasmin from the C6 glioma cells (lane 1), but did not recognize ceruloplasmin purified from serum (5-fold excess; lane 2). Control blot in lane 3. Molecular size markers from the top are 200 and 116 kDa.

FIG. 4. Ceruloplasmin is GPI-anchored on C6 glioma cells. Western blotting for the GPI anchor is shown. GPI-anchored proteins were cleaved from the surface of C6 glioma cells by PI-PLC treatment. Ceruloplasmin was then purified by mAb 1A1 immunoaffinity chromatography and Western blotted using an antibody that specifically recognizes PI-PLC-released GPI-anchored proteins (anti-CRD; Oxford Glycosystems). The antibody strongly recognized ceruloplasmin from the C6 glioma cells (lane 1), but did not recognize ceruloplasmin purified from serum (5-fold excess; lane 2). Control blot in lane 3. Molecular size markers from the top are 200 and 116 kDa.
The cell via a newly described iron transporter called Ftr1 (6). Whether the GPI-anchored form of ceruloplasmin in the mammalian central nervous system also functions along with a transporter similar to Ftr1 to transport ferric (Fe(III)) iron from the extracellular compartment into astrocytes is not known at present. Such a mechanism may contribute, along with other non-transferrin uptake systems (1, 7), to the influx of iron into astrocytes, which lack both the transferrin receptor (46, 47) and melanotransferrin (48). It is also possible that the egress of ferrous (Fe(II)) iron from neurons, oligodendrocytes, and astrocytes may occur via some as yet unidentified transporter, such as that proposed for other mammalian cells (3). The Fe(II) iron exiting these cells could become oxidized by the GPI-anchored form of ceruloplasmin on the surface of astrocytes. Since astrocyte processes are distributed throughout the central nervous system, ceruloplasmin located on astrocytes is ideally positioned to effectively oxidize the highly toxic ferrous iron to the ferric form. The latter may then be available for reutilization or cleared from the central nervous system by binding to transferrin or melanotransferrin.

Besides aceruloplasminemia, iron deposition has been observed in the substantia nigra in Parkinson’s disease (49, 50), in the cortex and amyloid plaques in Alzheimer’s disease (51, 52), in amyotrophic lateral sclerosis (53, 54), and in Hallervorden-Spatz disease (55). Evidence that iron may contribute to the neurodegeneration in these diseases is provided by the 6-hydroxydopamine-lesioned rat model of Parkinson’s disease, in which the iron chelator desferoxamine protects neurons from injury (56). Increased levels of free radicals, which may underlie the neurodegeneration, have been reported in the brains of Alzheimer’s and Parkinson’s patients (57–60). Reduction in ceruloplasmin has been reported in the cortex of patients with Alzheimer’s disease (61). It is possible that damage to astrocytes, resulting in reduced levels of the GPI-anchored form of ceruloplasmin in the affected gray matter regions, might lead to the iron deposition and free radical generation that causes the neuronal degeneration seen in these diseases. This is further supported by the finding that the ferroxidase activity of ceruloplasmin has been shown to inhibit ferrous iron-catalyzed phospholipid peroxidation in vitro (42, 43, 62, 63). The GPI-anchored form of ceruloplasmin on astrocytes which has this important ferroxidase activity may regulate iron transport in out of neurons and glia in the central nervous system, and may help limit lipid peroxidation in a tissue that is highly susceptible to oxidative injury.

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