Involvement of Glycosylphosphatidylinositol-linked Ceruloplasmin in the Copper/Zinc-Nitric Oxide-dependent Degradation of Glypican-1 Heparan Sulfate in Rat C6 Glioma Cells*

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The core protein of glypican-1, a glycosylphosphatidylinositol-linked heparan sulfate proteoglycan, can bind Cu(II) or Zn(II) ions and undergo S-nitrosylation in the presence of nitric oxide. Cu(II)-to-Cu(I)-reduction supports extensive and permanent nitrosotiol formation, whereas Zn(II) ions appear to support a more limited, possibly transient one. Ascorbate induces release of nitric oxide, which catalyzes deaminative degradation of the heparan sulfate chains on the core protein. Although free Zn(II) ions support a more limited degradation, Cu(II) ions support a more extensive self-pruning process. Here, we have investigated processing of glypican-1 in rat C6 glioma cells and the possible participation of the copper-containing glycosylphosphatidylinositol-linked splice variant of ceruloplasmin in nitrosotiol formation. Confocal microscopy demonstrated colocalization of glypican-1 and ceruloplasmin in endosomal compartments. Ascorbate induced extensive, Zn(II)-supported heparan sulfate degradation, which could be demonstrated using a specific zinc probe. RNA interference silencing of ceruloplasmin expression reduced the extent of Zn(II)-supported degradation. In cell-free experiments, the presence of free Zn(II) ions prevented free Cu(II) ion from binding to glypican-1 and precluded extensive heparan sulfate autodegradation. However, in the presence of Cu(II)-loaded ceruloplasmin, heparan sulfate in Zn(II)-loaded glypican-1 underwent extensive, ascorbate-induced degradation. We propose that the Cu(II)-to-Cu(I)-reduction that is required for S-nitrosylation of glypican-1 can take place on ceruloplasmin and thereby ensure extensive glypican-1 processing in the presence of free Zn(II) ions.

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Most of the intracellular copper ions are bound to proteins (15). Although the Gpc-1 core protein can directly bind both Cu(II) and Cu(I) (14), copper ion redox cycles could also take place on other juxtaposed cuproproteins. We have recently provided evidence that the GPI-linked, copper-binding cellular prion protein (PrP<sup>C</sup>), which colocalizes with Gpc-1 in T24 cells, can support an ascorbate-induced, NO-dependent HS degradation in Gpc-1 (14).

Ceruloplasmin (Cp) is a well known copper-binding protein with ferroxidase activity, mainly expressed and secreted by hepatocytes. However, astrocytes express a GPI-linked, cell-bound splice variant that is the major Cp in the brain (16). Rat C6 glioma cells also express GPI-linked Cp (17). The GPI-linked form arises by alternative splicing of Cp pre-mRNA such that the C-terminal 5 amino acids of the secreted form are replaced with an alternative 30 amino acids comprising the GPI signal in the membrane-bound form (17). The copper binding and ferroxidase properties of the secreted and the membrane-bound Cp should thus be the same. Accordingly, individuals with a hereditary deficiency of ceruloplasmin as well as Cp knock-out mice have severe iron deposition both in the liver and in the brain (18, 19).

Here, we have examined whether the GPI-linked Cp and Gpc-1 colocalize in C6 glioma cells and investigated how copper-loaded Cp and free zinc ions participate in the NO-dependent autodegradation of HS in Gpc-1. The results show that the GPI-linked Cp and Gpc-1 colocalize in intracellular compartments and that Cu(I) ion-loaded Cp can support extensive HS degradation even in the presence of free zinc ion.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rat C6 glioma cells were obtained from ATCC and maintained in Ham’s F12K serum supplemented with 15% horse serum and 2.5% fetal calf serum. Antisera raised against human or mouse Gpc-1, caveolin-1, mAbs recognizing SNO-Gpc-1 (mAb S1), a GlcNH<sub>2</sub><sup>−</sup>-containing HS epitope (mAb JM-403), anMan-terminating HS oligosaccharides (mAb AM), and suitably tagged secondary antibodies, as well as α-difluoromethylornithine (DFMO), brefeldin A, radioactive precursors, enzymes, prepacked columns, Centriplus tubes, and chemicals were generated or obtained as described previously (12–14). The antibody recognizing GPI-linked Cp (mAb 1A1) was described elsewhere (16). The zinc probe FluoZin-3 was obtained from Molecular Probes, iminodiacetic acid was from Fluka, and ceruloplasmin and glutathione were from Sigma.

[3H]Glucosamine-labeled Gpc-1 was obtained from DFMO- and brefeldin A-treated C6 glioma cells as described previously for T24 cells (12). The DFMO treatment was used to increase the content of GlcNH<sub>2</sub><sup>−</sup> units in HS and was carried out while cells grew to confluence, which usually took 1–2 days. The brefeldin A treatment was used to arrest recycling Gpc-1 at the stage prior to S-nitrosylation (13). Gpc-1 or Cp were saturated with divalent cations by exposure to 1 mM CuCl<sub>2</sub> or ZnCl<sub>2</sub> and then dialyzed against phosphate-buffered saline, pH 7.4. Cu(II)-loaded Cp was also exposed to 1 mM FeSO<sub>4</sub> followed by dialysis.

**siRNA Preparation and Transfection**—The vector pRNA-U6.1/Neo (Genescript Corporation) containing the sequence TTCAACAAAGGC-GAACGA (corresponding to nucleotides 374–392 in rattus norvegicus GPI-anchored ceruloplasmin) followed by a hairpin sequence TTCAA-

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**Scheme 1. Model of Gpc-1 that is S-nitrosylated in a Cu(II)-dependent reaction, releasing NO upon exposure to ascorbate and cleaving its own HS chains. In the top left, Gpc-1 proteoglycan with thiol (SH) groups in the globular part of the protein core (circle) and substituted with three HS chains (unbroken, long thick lines) extending from a segment near the GPI-anchor (filled small circle) has bound Cu(II). Upon exposure to NO, an SNO and Cu(I)-containing Gpc-1 is formed (top right). When this in turn is exposed to ascorbate, NO is released, ascorbate is presumably oxidized to dehydroascorbate and thiols are reformed, Cu(I) is reoxidized to Cu(II), and the released NO is reduced to nitroxyl ion (NO<sup>−</sup>) (bottom right). The concentration of NO<sup>−</sup> in this microenvironment is sufficient to result in deaminative cleavage of the HS chains at N-unsubstituted glucosamines, generating anMan-terminating HS oligosaccharides (short thick lines), a Gpc-1 with truncated HS chains, and molecular nitrogen (bottom left). With a steady supply of NO and reducing agent, the cyclic process should continue until all available sites are cleaved.

**Scheme 2. Model of NO-dependent autocleavage of Gpc-1 HS chains in a Zn(II)-dependent reaction, involving transient formation of SNO groups. In the top left, Gpc-1 proteoglycan with SH groups in the globular part of the protein core (circle) and substituted with three HS chains (unbroken, long thick lines) contains bound Zn(II), which should form zinc-thiolate complexes. Zn(II) binding appears to preclude binding of Cu(II) ions. When exposed to NO, Gpc-1 is transiently S-nitrosylated (top right). When exposed to ascorbate, Gpc-1 releases NO and the redox-inert Zn(II) ions, the thiols are probably oxidized to disulfides, and nitroxyl ion (NO<sup>−</sup>) is formed (bottom right). Ensuing NO<sup>−</sup>-dependent deaminative cleavage of the HS chains in the same molecule generates HS fragments (broken thick lines) and Gpc-1 with truncated HS chains (bottom left). If the disulfides are not reduced to thiols, the process is terminated, and the extent of HS degradation remains limited.
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GAGA, then the reversed complementary ceruloplasmin sequence with a stretch of six T for RNA polymerase III termination followed by GGAAA in the 3’-end, was synthesized by Genscript Corporation. A negative control vector comprising a scrambled sequence was also prepared. Transfection was accomplished by using LipofectAMINE (Invitrogen) according to the description of the manufacturer.

Auto-cleavage of HS in Gpc-1—The high molecular weight Gpc-1 precursor (−1 mM) was exposed to various combinations of metal ions, Cp, NO donor (sodium nitroprusside (SNP)), and triggered by ascorbate (1 mM for 10 min) to autodegrade its own HS chains, all in phosphate-buffered saline, pH 7.4. The reactions were monitored by Superose 6 gel chromatography of the various NO-released HS fragments and the alkali-released stubs remaining on the core protein as described previously (12). Control degradation of GlcNH$_3^+$ units by HNO$_2$ at pH 3.9 was carried out as described (14).

Confocal Laser Scanning Immunofluorescence Microscopy—The various procedures including seeding of cells, fixation, the use of primary and secondary antibodies, generation of images by sequential scans, and data processing were the same as those used previously (12–14). Antibodies raised against human Gpc-1 core protein$_{14-558}$ or mice Gpc-1 core protein$_{12920-13554}$ cross-react with rat and hamster Gpc-1 (20). The secondary antibodies used were either goat anti-mouse total Ig (when the primary antibody was a mAb) or goat anti-rabbit IgG (when the primary antibody was a polyclonal antiserum). They were tagged with either fluorescein isothiocyanate or Texas Red and appropriately combined for colocalization studies.

RESULTS

GPI-linked Cp Colocalizes with Gpc-1, and Ascorbate Induces HS Cleavage at GlcNH$_3^+$ in Glioma Cells—As shown in Fig. 1, A–C, Gpc-1 and GPI-linked Cp colocalized in intracellular compartments of rat C6 glioma cells. SNO forms of Gpc-1, detected by mAb S1, and caveolin-1 did not colocalize (Fig. 1, D–F). However, the S1 signal was relatively weak compared with that from total Gpc-1 (cf. Fig. 1, G and H), resulting in a very faint colocalization signal in the merged panel (Fig. 1I). Thus, Gpc-1 appeared to be mainly in a non-nitrosylated state. Many of the Gpc-1 molecules were substituted with GlcNH$_3^+$-containing HS chains (mAb JM-403), as indicated by the colocalization experiment shown in Fig. 1, J–L.

Treatment of cells with ascorbate should induce NO release from stable SNO groups in Gpc-1 and/or support zinc-dependent transnitrosation of Gpc-1 with subsequent cleavage of HS at GlcNH$_3^+$ in both cases (Schemes 1 and 2) (see also Refs. 12–14). The results showed that ascorbate had no effect on total Gpc-1 (cf. Fig. 1, J and M), whereas the signal from the JM-403 HS epitope was reduced (cf. Fig. 1, K and N), indicating HS degradation at the GlcNH$_3^+$ sites. However, the S1 epitope was not markedly affected (cf. Fig. 1, H and O), suggesting that NO and nitroxy anion were not primarily derived from stable SNO groups in Gpc-1 but perhaps via Zn(II)-supported transnitrosation.

Zn(II) Ion-supported Extensive, NO-dependent, Ascorbate-Induced Degradation of HS Depend on Expression of Cp—To investigate Zn(II)-supported HS degradation in glioma cells, we used a divalent metal ion chelator (iminodiacetate) supplemented with ZnCl$_2$. In the control experiment, cells were preincubated with 1 mM iminodiacetate for 1 h to prevent S-nitrosylation/denitrosylation and then treated with 1 mM ascorbate (Fig. 2, A and B). The signals for Gpc-1 (results not shown) and GlcNH$_3^+$-containing HS (JM-403) were largely unaffected (cf. Fig. 1K with Fig. 2A), suggesting minimal HS degradation.

However, when cells that had been preincubated with 1 mM iminodiacetate and 1 mM ZnCl$_2$ for 1 h were exposed to 1 mM...
ascorbate for 1 h, there was extensive degradation of HS as indicated by a significant reduction of the JM-403 signal (Fig. 2E). Simultaneously, there was an appearance of anMan-positive HS degradation products (cf. Fig. 2, B and F).

We then tested the effect of Cp-specific RNA interference silencing on Zn(II)-supported, deaminative cleavage of HS. In mock-transfected (Fig. 2C) cells, Zn(II)-supported HS degradation was still going on and was similar to that in untreated cells (cf. Fig. 2, D and E). In cells transfected with a Cp-specific siRNA-producing vector, the expression of GPI-Cp was silenced (cf. Fig. 2, C and G), and Zn(II)-supported HS degradation was markedly reduced (cf. Fig. 2, D and H) and similar to that in cells not exposed to Zn(II) ion (Fig. 2A).

Zn(II) Ion Release from Gpc-1 during Ascorbate-triggered Degradation of HS—In the course of the transnitrosation reaction assumed to be involved in zinc-supported, NO-dependent deaminative cleavage of HS, Zn(II) ions should be released from Gpc-1 when ascorbate triggers NO release, nitroxylin anion formation, and HS degradation (14). We first tested the formation of SNO groups by using mAb S1, which is specific for this Gpc-1 form (13). Cells treated with NO donor and Zn(II) ions had increased content of SNO-Gpc-1 (cf. Figs. 1H and 3A). Addition of ascorbate eliminated the signal almost completely (Fig. 3B). To investigate Zn(II) ion release, we used a sensitive, specific, and fluorescent zinc probe. When the probe was used at a concentration of 2.5 μM in all of the experiments, free Zn(II) ions were barely detectable in unperturbed cells (Fig. 3C) and almost undetectable after exposure to NO donor alone, in keeping with an increased binding of Zn(II) ions to Gpc-1 (Fig. 3D). The level of free Zn(II) ion increased slightly after exposure to ascorbate alone (cf. Fig. 3, C and E) but increased significantly after exposure to both NO donor and ascorbate (cf. Fig. 3, D and F). The released free Zn(II) ions colocalized with Gpc-1 at paranuclear sites (results not shown) and, simultaneously, there was generation of anMan-containing HS oligosaccharides (Fig. 3G), which partially colocalized with Gpc-1 (Fig. 3H, yellow).

Taken together, the preceding results suggested that there was extensive zinc-supported, NO- and Cp-dependent HS degradation in glioma cells. However, in previous cell-free experiments, zinc-supported HS degradation was relatively limited (14). We therefore investigated how the presence of Cp affected zinc-supported, deaminative degradation of HS in cell-free experiments.

The Mechanism of Cp-supported Autocleavage of HS in Zn(II)-Loaded Gpc-1—In these experiments, we used a purified Gpc-1 that was [3H]GlcN-labeled in the HS chains and isolated from polyamine-deprived and brendielin A-treated C6 glioma cells. As shown previously (12–14), this gives rise to a Gpc-1 glycoform with long and GlcNH$_3^+$-rich HS chains. The intact glioma-derived Gpc-1 PG was excluded from Superose 6 upon gel exclusion chromatography (Fig. 4A), and the HS chains released by alkaline borohydride treatment were partially included on the same gel (Fig. 4B). There was extensive depolymerization of the HS chains upon treatment with HNO$_2$ at pH 3.9, resulting in a much retarded elution position on Superose 6. This indicates a high content of GlcNH$_3^+$ residues (Fig. 4C).

When such intact Gpc-1 PG, loaded with Zn(II) ion, was exposed to NO donor alone, there was no sign of HS degradation (Fig. 4D). However, when ascorbate was included, there was a limited depolymerization of the HS chains (Fig. 4E), in keeping with previous results (14). In contrast, there was a more extensive degradation of HS when Gpc-1 was first S-nitrosylated by pre-exposure to Cu(II) ion and NO donor and then treated with ascorbate (Fig. 4F). As shown previously (14), when the Gpc-1 PG contained bound Zn(II) ions, extensive, Cu(II) ion-supported HS degradation was prevented (Fig. 4G).

In contrast, when Cu(II)-containing Cp and Zn(II)-loaded Gpc-1 were exposed to NO donor and ascorbate, the HS chains of Gpc-1 underwent extensive degradation (Fig. 4H). The extent of degradation was almost the same as that obtained with free Cu(II) ion (Fig. 4F). This extensive degradation was independent of Zn(II) ions (Fig. 4I) but dependent on the presence of ascorbate (Fig. 4J). When a mixture of Zn(II)-loaded Gpc-1 and Cu(II)-containing Cp that was pre-exposed to Fe(II) ion was treated with NO donor, with or without ascorbate, the HS chains were only marginally degraded (Figs. 4, K and L). Finally, we tested the effect of glutathione. When 1 mM glutathione was included in a reaction mixture containing Cu(II)-loaded Cp, HS degradation was completely inhibited (results not shown).

**DISCUSSION**

The present results show that the GPI-anchored splice variant of the cuproprotein Cp colocalizes with the similarly GPI-linked HSPG Gpc-1 in intracellular compartments of rat C6

![Fig. 3. Transient SNO formation, Zn(II) ion-release and HS oligosaccharide formation from Gpc-1 upon stimulation with ascorbate.](image-url)
glioma cells. In these cells, ascorbate induces an NO-dependent autodegradation of the HS chains in Gpc-1. The nitroxy anion required for cleavage of HS at the GlcNH₃/H₁₁₁₀₁ residues can be generated either by denitrosylation of preformed SNO groups in Gpc-1 or by Zn(II) ion- and NO-dependent transnitrosation of Gpc-1. Stable SNO formation is dependent on a Cu(II)/Cu(I) redox cycle (12). In cell-free experiments, Zn(II) ion prebound to Gpc-1 precludes copper-supported HS degradation, probably because the two cations bind to the same site(s) in the Gpc-1 core protein (14). Moreover, in cell-free experiments, zinc-supported degradation is less extensive than copper-supported degradation. However, in C6 cells, the ascorbate-induced HS degradation was quite extensive, despite the fact that it appeared to be mainly zinc-supported. This was apparently dependent on the expression of Cp in these cells.

Cp binds seven copper atoms, three mononuclear ones and a trinuclear copper center that confers ability to serve as a ferroxidase. An additional copper is bound to the surface of the molecule near His-426. All of the copper atoms except one can be in either a Cu(II) or a Cu(I) state. Under normal conditions extracellularly, Cp binds Fe(II), oxidizes it, and loads Fe(III) on to transferrin. However, within the intracellular compartments or under the cell-free conditions used here, the copper in Cp may be available for other Cu(II)-mediated reactions. This may involve only the copper bound at His-426 or also other copper atoms in Cp (21–23).

As shown here, GPI-Cp is another potential regulator of NO-dependent degradation of HS in Gpc-1. In cell-free experiments and in the presence of Cu(II)-loaded Cp, HS undergoes extensive degradation also in Zn(II)-loaded Gpc-1 exposed to NO donor and ascorbate. Hence, as shown in Scheme 3, in the presence of Cp, the limitations imposed by Zn(II) ions are
overcome (cf. Scheme 2). The Cp-Cu(II) involved in S-nitrosylation of Gpc-1 should be reduced to Cu(I). As Cu(II)-loaded 
Cp treated with excess Fe(II) ions was ineffective, it suggests that the presence of Cu(II) ions in Cp is essential.

It should be added that Cu(II)-loaded Cp has been shown to catalyze S-nitrosylation of glutathione (24). This may explain why glutathione inhibited Cp-supported HS degradation; i.e. NO was used to nitrosylate glutathione rather than Gpc-1.

Although Cu(I)-containing Cp may release NO, conversion of the free radical NO to nitroxy anion is required for the degradation of HS at the GlcNH$_3^+$ residues (12–14). It should be pointed out that ascorbate is actively taken up by astrocytes (25) and could thus contribute to nitroxy anion formation and subsequent HS degradation in glioma cells. Other studies have demonstrated that Gpc-1 core protein can be detected in the nuclei of neurons, glia cells, and Chinese hamster ovary cells (6, 20). The nuclear appearance of Gpc-1 in C6 glioma cells varied with different phases of the cell cycle, suggesting that Gpc-1 could be involved in the regulation of cell division (6). In Chinese hamster ovary cells, the nuclear appearance of Gpc-1 was induced by ascorbate (20). Interestingly, ascorbate has been shown to promote differentiation of neural stem cells into neurons and astrocytes while inducing expression of differentiation-specific genes (26). It is thus possible that this is due to nuclear targeting of Gpc-1 core protein rather than to a direct effect of ascorbate.

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