Copper are generally bound to proteins, e.g. the prion and the amyloid β proteins. We have previously shown that copper ions are required to nitrosylate thiol groups in the core protein of glypican-1, a heparan sulfate-substituted proteoglycan. When S-nitrosylated glypican-1 is then exposed to an appropriate reducing agent, such as ascorbate, nitric oxide is released and autocatalyzes deaminative cleavage of the glypican-1 heparan sulfate side chains at sites where the glucosamines are N-unsubstituted. These processes take place in a stepwise manner, whereas glypican-1 recycles via a caveolin-1-associated pathway where copper ions could be provided by the prion protein. Here we show, by using both biochemical and microscopic techniques, that (a) the glypican-1 core protein binds copper(II) ions, reduces them to copper(I) when the thiols are nitrosylated and reoxidizes copper(I) to copper(II) when ascorbate releases nitric oxide; (b) maximally S-nitrosylated glypican-1 can cleave its own heparan sulfate chains at all available sites in a nitroxylnon-dependent reaction; (c) free zinc(II) ions, which are redox inert, also support autocleavage of glypican-1 heparan sulfate, probably via transnitrosation, whereas they inhibit copper(II)-supported degradation; and (d) copper(II)-loaded but not zinc(II)-loaded prion protein or amyloid β peptide support heparan sulfate degradation. As glypican-1 in prion null cells is poorly S-nitrosylated and as ectopic expression of cellular prion protein restores S-nitrosylation of glypican-1 in these cells, we propose that one function of the cellular prion protein is to deliver copper(II) for the S-nitrosylation of recycling glypican-1.

Heparan sulfate (HS)³ chains are known to be associated with amyloid deposits, including those generated in neurodegenerative disorders like prion and Alzheimer’s diseases. The HS component appears to be critical both for the deposition and the persistence of the cytotoxic protein aggregates (1–5). The proteins forming the aggregates are Cu- and Zn-binding proteins, and metal ions have also been implicated in the disease process (for review, see Refs. 6 and 7). HS does not occur normally as free glycan chains but is linked covalently to proteins forming proteoglycans (PG). How HS is released, from which PG it is derived, and how it binds to the aggregating proteins have not been clarified.

The glycosylphosphatidylinositol (GPI)-anchored cellular prion protein (PrP°C) binds Cu(II) ions to five sites and Zn(II) ions to one or two sites in the N-terminal domain (6, 8–10). Cu(II) ions appear to be preferred, and binding of Cu(I) has not been observed (11). PrP°C also binds to HS, which interacts with the Cu(II)-containing domain in PrP°C and weakens the affinity for Cu(II) (12–15). The amyloid β protein precursor as well as the amyloid β peptide (Aβ) generated from it can also bind both Cu(II) and Zn(II) ions (16–19). In this case, Cu(II) can be reduced to Cu(I) and remain bound, whereas the Zn(II)-binding motifs appear to constitute the HS-binding sites (16, 19).

Cu(II) binding to PrP°C stimulates endocytosis and recycling of PrP°C (20, 21). During recycling via caveolae-dependent internalization, PrP°C can be converted to the abnormal, aggregation-prone scrapie isoform (PrP°Sc) facilitated by the presence of a protein X (6). HS affects the conversion of PrP°C to PrP°Sc in a complex manner (for review, see Ref. 22). Although HS can inhibit conversion in a cell-free system, in the context of endocytosis, there appears to be stimulation. In contrast, aggregation of the Aβ is induced by Zn(II) ions (7, 17) and can be inhibited by metal ion chelators (18). The formation of the Aβ takes place in the Golgi and in endosomes (for review, see Ref. 23).

We have previously studied a recycling, HS-substituted, GPI-linked PG named glypican-1 (Gpc-1) (24–28). Gpc-1 belongs to a family of HS-containing PG with six known mammalian members to date. They are common in the brain and many other tissues and are present mainly at the cell surface, probably in rafts and caveolae (for reviews, see Refs. 29–31). Gpc-type PG have a characteristic pattern of 14 conserved cysteine residues in the central domain. The attachment sites for 2–4 HS-chains are located near the GPI anchor. The HS chains consist of a repeating disaccharide backbone of glucosamine and hexuronic acid that is regionally and variably modified by epimerization of the hexuronic acid and by different types of sulfations on both sugars, creating a variety of binding sites for polycationic molecules, such as polyanimes and basic peptide growth factors. Gpc-1, which recycles via caveolin-1-positive endosomes, is thus a potential membrane receptor for the transport of cargo into and through cells (24–28). Shortly after.
after internalization, Cys residues in the Gpc-1 core protein become S-nitrosylated (27). The S-nitroso groups (SNO) of Gpc-1 appear to remain stable until Gpc-1 is exposed to a suitable reducing environment where nitric oxide (NO) is released and cleaves the intrinsic HS chains at glucosamine units lacking N-substitution (GlcNH\(\text{2}^-\)) (25). At steady state, there are both Gpc-1 molecules with truncated HS chains and free HS oligosaccharides inside the cells (24). NO-dependent autocleavage of recycling Gpc-1 is necessary for uptake and delivery of polyanymes, small basic molecules that are essential for cell growth and survival (26–28).

S-Nitrosylation of Gpc-1 and subsequent NO release are dependent on a Cu(II)-Cu(I) redox cycle (27). We have postulated (Fig. 1) that when the Gpc-1 core protein is S-nitrosylated, Cu(II) is reduced to Cu(I). NO release from the S-nitroso groups can be induced by ascorbate. Nitroxyl anion (NO\(-\)) could then be formed from NO, whereas Cu(I) is oxidized to Cu(II). In the present study, we have investigated copper binding to Gpc-1 and the nature of the HS-cleaving NO species. We demonstrate that both Cu(II)-loaded PrP\(\text{C}\) and Aβ can deliver copper ions to Gpc-1 to support S-nitrosylation, which is a prerequisite for nitroxyl ion-dependent HS-degradation. Free Zn(II) ions, which are redox inert, also indirectly support HS degradation but by a different mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—The human bladder carcinoma cell-line T24, culture media, antiserum to human or mouse Gpc-1, SNO-Gpc-1 (S1), SNO-cysteine (SNO-Cys), a GlcNH\(\text{3}^-\)-containing HS epitope (JM-403), caveolin-1, and suitably tagged secondary antibodies, as well as \(\alpha\)-difluoromethyloxirane (DFMO), brefeldin A (BFA), radioactive precursors, enzymes, prepacked columns, Centriplus tubes, and chemicals were generated or purchased from Prionics, Zurich, Switzerland, Apc-1, mouse Gpc-1 was a generous gift from Prof. G. David, University of Leuven, Belgium. Embryonic fibroblasts from prion null mice (PRNP0/0) were obtained as described previously (24–28). An antibody was polyclonal). They were tagged with either fluorescein (when the primary antibody was a monoclonal) or goat anti-rabbit IgG (when the primary antibody was polyclonal). These antibodies included seeding of cells, fixation, the use of primary and secondary antibodies, generation of images, and data processing were the same as those used previously (27, 28). The secondary antibodies were used with goat anti-mouse total Ig (when the primary antibody was a monoclonal) or goat anti-rabbit IgG (when the primary antibody was polyclonal). They were tagged with either fluorescein isothiocyanate or Texas Red and combined appropriately for colocalization studies.

**RESULTS**

**The Mechanism of Copper-supported Autocleavage of HS in Gpc-1**—We demonstrated previously that Cu(II) ions, NO donor, and ascorbate are all needed to sustain autocleavage of HS in purified Gpc-1 (see Fig. 1 and Ref. 27). Preloading of Gpc-1 with Cu(II) ions followed by dialedysis did not affect its ability to become S-nitrosylated, suggesting the presence of copper-binding sites in the Gpc-1 protein core. Furthermore, S-nitrosylated Gpc-1 was quite stable in the absence of reducing agents in accordance with the presence of SNO-containing Gpc-1 molecules in unperturbed T24 cells (28). Upon ascorbate treatment of SNO-containing, immunopurified Gpc-1, NO was rapidly released, and the HS chains were cleaved at the GlcNH\(\text{3}^-\) units, suggesting that NO\(-\) could be the reactive species (27).

The number of GlcNH\(\text{3}^-\) units in the HS chains of Gpc-1 can be increased by inhibiting both recycling and endogenous polyamine synthesis (26). Most of the GlcNH\(\text{3}^-\) units of HS derived from recycling Gpc-1 of unperturbed cells are located close to the linkage to protein (25), whereas those units that are generated upon BFA arrest and polyamine deprivation are also located at multiple sites along the chain (26). The presence of GlcNH\(\text{3}^-\) units can be demonstrated by cleavage with nitrite at pH 3.9 followed by gel chromatography. As shown in Fig. 2A (filled circles), HS from Gpc-1 arrested at the precursor stage by treating cells with BFA was degraded by nitrite under these conditions. Corresponding HS from cells treated with both BFA and DFMO was more degraded (Fig. 2D, filled circles). According to previous estimates (25), this degradation should correspond to an average size reduction from ~100 kDa to 50 kDa (Fig. 2A) and 30 kDa (Fig. 2D), respectively.

NO release requires a reducing agent. We have previously used ascorbate (27). Here we also tested glutathione, an abundant intracellular reducing agent. However, 1 mM glutathione was unable to initiate HS degradation (Fig. 2B, open diamonds), whereas 1 mM ascorbate generated cleavage at nearly all of the GlcNH\(\text{3}^-\) units as monitored by gel chromatography (cf. Fig. 2, A and B, filled symbols).

The capacity to generate NO for HS degradation should be dependent on the level of S-nitrosylation. This, in turn, depends on the number of available thiol groups. Human Gpc-1 contains 17 Cys residues (32). Fourteen of these are conserved between various glypcans (29–31). The number of Cys residues involved in disulfide bonding has never been elucidated. The reduction of disulfides to thiols should increase the number
FIG. 2. Copper ion-supported autocleavage of HS in S-nitrosylated Gpc-1 monitored by chromatography on Superose 6. A, chromatogram of intact [3H]HS chains derived from Gpc-1 produced by BFA-treated T24 cells (open circles) and of the same HS chains treated with HNO₂ at pH 3.9, which specifically cleaves at GlcNH₃⁺ (filled circles). B, HS chains and oligosaccharides derived from the same Gpc-1 as in A, after
of available nitrosylation sites. We tested this by treating purified Gpc-1 with dithiothreitol prior to S-nitrosylation. Subsequent treatment with ascorbate induced a very extensive degradation of some of the HS chains (Fig. 2C). According to previous estimates (25) this should correspond to a size reduction from ~100 to 20 kDa, i.e. cleavage at four sites/HS chain on average.

S-Nitrosylation of Gpc-1 requires Cu(II) ions (27). Hence, the number of SNOs in Gpc-1 should also be regulated by the availability of copper ions. We therefore exposed purified Gpc-1 to increasing concentrations of Cu(II) ions prior to treatment with NO donor and ascorbate (Fig. 2E). When exposure to copper ion was omitted, there was no degradation (Fig. 2E, open circles). With exposure to increasing concentrations of Cu(II) ions, there was an increased degradation of HS (Fig. 2E, filled symbols). Preincubation with an excess of Cu(II) ions caused the greatest degradation (Fig. 2E, filled diamonds). This degradation should also correspond to ~4 cleavages/chain.

When the capacity for S-nitrosylation was increased either by the generation of more thiol (Fig. 2C) or by providing an excess of Cu(II) ions (Fig. 2E), the extent of HS degradation appeared to slightly exceed that obtained by cleavage only at the GlcNH₃⁺ units (cf. Fig. 2, A and D, filled symbols). To test whether there was additional cleavage at N-sulfated glucosamine, a [³H]glucosamine- and [³⁵S]sulfate-labeled Gpc-1 preparation was S-nitrosylated in the presence of excess Cu(II) ion and then exposed to ascorbate (Fig. 2F). A peak in the expected position of free sulfate was seen (open squares).

There are three different NO species: the positively charged nitrosium ion (NO⁻⁺), the uncharged NO radical (NO⁻), and the negatively charged nitroxy anion (NO⁻⁻) (for review, see Ref. 33). The proportions depend on the redox state of the environment. NO⁻ should be attracted by the GlcNH₃⁺ residues and could therefore be the reactive species in HS degradation. As cysteine is known to react with NO⁻⁻ (34), we performed the NO release reaction with ascorbate in the presence of an excess of cysteine (Fig. 2G). Degradation of HS in SNO-containing and ascorbate-exposed Gpc-1 was markedly reduced in the presence of cysteine, confirming that NO⁻ should be the major reactive species.

The Gpc-1 core protein contains several His residues that could form Cu(II)-binding sites (32). Cys residues could form Cu(I)-binding sites. To investigate copper binding to Gpc-1 we used affinity chromatography (Fig. 3). As a control we applied ¹²⁵I-labeled PrP with Cu(I)-loaded HiTrap chelating matrix. As expected, PrP with Cu(I)-loaded material was displaced with elution with 50 mM iminoacetate (Fig. 3A). No binding occurred to unloaded HiTrap, and binding to Cu(I)-loaded HiTrap diminished when PrP was applied in the presence of 1 mM CuCl₂. We then tested Gpc-1 binding to a Cu(I)-loaded matrix. As shown in the model (Fig. 3B), binding should not be inhibited by Cu(I) ions, but when NO donor is provided, binding should be diminished, if Cu(I) at Gpc-1 is reduced to Cu(I) during S-nitrosylation of Gpc-1 (27). Binding should then be restored when ascorbate is added, because this should result in NO release and reoxidation of Cu(I) to Cu(II). As shown in Fig. 3C, this is exposure to 1 mM SNP and 1 mM CuCl₂ followed by dialysis and treatment with 1 mM reduced glutathione (open diamonds) or 1 mM l-ascorbate (filled diamonds). C, HS chains and oligosaccharides derived from the same Gpc-1 as in A, after exposure to 1 mM dithiothreitol, dialysis, and exposure to 1 mM SNP and 1 mM CuCl₂ followed by dialysis and treatment with 1 mM l-ascorbate. D, chromatogram of intact [³H]HS chains derived from Gpc-1 produced by BFA- and DFMO-treated T24 cells (open circles) and of the same HS chains treated with HNO₂ at pH 3.9, which specifically cleaves at GlcNH₃⁺ (filled circles). E, HS chains and oligosaccharides derived from the same Gpc-1 as in D, after exposure to 1 mM SNP in the absence (open circles) or presence of 10 µM CuCl₂ (filled circles) or 100 µM CuCl₂ (filled squares) or after S-nitrosylation of Gpc-1 that was presaturated with copper ion by dialysis in the presence of 1 mM CuCl₂ (filled diamonds), all followed by treatment with 1 mM l-ascorbate. F, [³H]HS chains and oligosaccharides derived from Gpc-1 produced by BFA- and DFMO-treated cells labeled with both [³H]glucosamine and [³⁵S]sulfate that was saturated with Cu(II) ion, S-nitrosylated, and treated with ascorbate (H, filled diamonds; ³⁵S, open squares). G, HS chains from the same Gpc-1 as in D, after pretreatment of Gpc-1 with Cu(II) ion (as in E, filled diamonds) and S-nitrosylation followed by treatment with 1 mM l-ascorbate in the presence of 3 mM l-cysteine.
precisely what happened. It should be added that the thiol-containing Cu(I)-loaded matrix binds insignificant amounts of PrPC (< 5%).

The Mechanism of Zinc-supported Auto cleavage of HS in Gpc-1—Purified Gpc-1 was exposed to Zn(II) ions and NO donor in an attempt to generate S-nitrosylated Gpc-1 (Fig. 4A). The Gpc-1 preparation that was exposed to Zn ion and NO donor simultaneously and then recovered after dialysis was poorly degraded upon the addition of ascorbate (cf. open and filled circles in Fig. 4A). However, in a Gpc-1 preparation that was first exposed to Zn(II) ions, then dialyzed, and subsequently treated with NO donor and ascorbate simultaneously, there was a partial HS degradation (Fig. 4B, filled circles). The omission of ascorbate precluded HS degradation (Fig. 4C).

In the case of Cu(II)-supported HS degradation (Fig. 2), prior reduction of disulfides greatly increased the capacity for HS degradation (Fig. 2C). This was not the case with Zn(II)-supported HS degradation (Fig. 4D), suggesting that the mechanism might be different. However, the reactive NO form appeared to be NO−, because cysteine inhibited Zn(II)-supported HS degradation (Fig. 4E). We also used an especially GlcNH2-rich Gpc-1 preparation obtained from cells that were treated with both BFA and DFMO. As shown in Fig. 4F (filled circles), Zn(II)-supported HS degradation in this preparation was quite...
extensive, although it was not as extensive as cleavage at all available sites by HNO₂ at pH 3.9 (cf. Fig. 4F, open circles). Hence, Zn(II)-supported autocleavage was less extensive than Cu(II)-supported cleavage (cf. Fig. 4B, filled circles with Fig. 2B, filled diamonds). When Gpc-1 was dialyzed in the presence of equimolar amounts of Zn(II) and Cu(II) ions before exposure to NO donor, the extent of ascorbate-induced HS degradation was markedly reduced (Fig. 4B, filled diamonds) compared with Cu(II)-only-supported autocleavage (Fig. 2B, filled diamonds) and only marginally greater than that obtained with Zn(II) ions alone (Fig. 4B, filled circles). These results suggest that Zn(II) and Cu(II) ions partly compete for the same sites in Gpc-1 and therefore prebound Cu(II)-supported S-nitrosylation could preclude Cu(II)-supported S-nitrosylation.

Because Zn(II) is redox inert, other redox agents must be involved if S-nitrosylation were indeed taking place. To investigate whether stable SNOs were generated, we performed confocal immunofluorescence microscopy using monoclonal antibody S1, which is specific for S-nitrosylated Gpc-1 (28). S-Nitrosylation of Gpc-1 (Fig. 5A) was completely obliterated by treatment with ascorbate (Fig. 5B). When NO donor and Cu(II) were provided after ascorbate treatment, the SNO-containing Gpc-1 epitopes were partially restored (Fig. 5C). However, when Zn(II) was substituted for Cu(II), S-nitroso Gpc-1 could not be detected (Fig. 5D). Taken together, these results indicate that formation of stable SNOs is not required in connection with Zn(II)-supported HS-degradation in Gpc-1. However, there must be Zn(II)-binding sites in Gpc-1, as Zn(II)-treated and dialyzed Gpc-1 was able to autodegrade its HS chains upon exposure to NO donor and ascorbate (Fig. 4, B and F).

**PrP<sup>C</sup> and Aβ Involvement in S-Nitrosylation of Gpc-1—**As both PrP<sup>C</sup> and Gpc-1 are GPI-linked molecules they may colocalize in lipid rafts, caveolae, or caveosomes. To immunolocalize PrP<sup>C</sup>, Gpc-1, and caveolin-1 in T24 cells we used confocal immunofluorescence microscopy (Fig. 6, A and B). Colocalization of PrP<sup>C</sup> and Gpc-1 was negligible at the cell surface but prominent in vesicles located to para- and perinuclear sites (Fig. 6A, yellow). In the same area there was also colocalization between PrP<sup>C</sup> and caveolin-1 (Fig. 6B, yellow). At the cell surface, most of the caveolin-1 was at separate sites. As shown elsewhere (28), the Gpc-1 that colocalizes with caveolin-1, and thus also the Gpc-1 that colocalizes with PrP<sup>C</sup>, carries HS chains rich in the NO-sensitive GlcNH₃ residues. In these perinuclear vesicles, Gpc-1 also colocalizes with Rab9 and the
PrPC and Aβ-derived copper ions support autocleavage of HS in S-nitrosylated Gpc-1 as monitored by chromatography on Superose 6. A, chromatogram of [3H]HS chains and oligosaccharides derived from Gpc-1 produced by BFA- and DFMO-treated T24 cells, pre-exposed to PrPC (10 ng) that had been presaturated with 1 mM CuCl2 and then treated with 1 mM SNP followed by 1 mM L-ascorbate (filled symbols) and HS chains derived from the same Gpc-1 pre-exposed to both HS chains (100 ng) and Cu(II)-loaded PrPC followed by NO donor and ascorbate (open symbols). B, HS chains derived from the same Gpc-1 treated as in A (filled symbols), except that ZnCl2 was used instead of CuCl2. C, HS chains and oligosaccharides derived from Gpc-1 pre-exposed to Cu(II)-saturated Aβ (20 ng) and then treated with NO donor and ascorbate. D, HS chains derived from Gpc-1 pre-exposed to Zn(II)-saturated Aβ (20 ng) and then treated as in C.

**DISCUSSION**

Functional Interplay Between PrPC and Gpc-1—The available data thus suggest a mechanism for the S-nitrosylation of Gpc-1 by Cu(II)-loaded PrPC and NO. Non-S-nitrosylated Gpc-1 and PrPC loaded with Cu(II) could be transiently located together on the same membrane (Fig. 8A). The interaction between PrPC and the HS chains of Gpc-1 should result in the transfer of Cu(II) from PrPC to Gpc-1 (Fig. 8B). NO synthase (Fig. 8B, NOS), which is present at the cytosolic surface of the membrane, generates NO from Arg, and Gpc-1 becomes S-nitrosylated, whereas Cu(II) is reduced to Cu(I) (Fig. 8C). SNO-containing Gpc-1 should remain stable until ascorbate, or the unknown endogenous reducing agent, is encountered. Then NO is released, Cu(I) is reoxidized to Cu(II), NO− is generated, and HS is cleaved at the GlcNH3+ residues (Fig. 8D; see also Refs. 27 and 28).

The extent of HS degradation varies with the amount of NO released, which depends on the number of SNOs, which, in turn, depends on the number of available thiols. Maximum HS degradation appears to correspond to ~4 cleavages/chain on an

Rab9-containing vesicles are also caveolin-1-positive. Thus, PrPC and Gpc-1 with GlcNH3+-rich HS chains may colocalize to caveosomes or sorting endosomes.

PrPC is thus in a position where it could supply Gpc-1 with the Cu(II) ions required for S-nitrosylation. To examine whether PrPC is involved in S-nitrosylation of Gpc-1, we used embryonic fibroblasts derived from mice with a targeted disruption of the PrPC gene (Prnp<sup>−/−</sup>). As shown in Fig. 6, C–E, respectively, the prion null fibroblasts expressed mouse Gpc-1 (mGpc) and could S-nitrosylate proteins in general (SNO-Cys) but could not S-nitrosylate Gpc-1 (SI). However, when the prion null cells were treated with CuCl2 and sodium nitroprusside (SNP) as the NO donor, there was significant S-nitrosylation of Gpc-1 (Fig. 6F) indicating that prion null cells express a functional Gpc-1 protein. When prion null cells were transfected with cDNA for mouse PrPC, S-nitrosylation of Gpc-1 was restored (Fig. 6G) indicating that PrPC is specifically involved in the S-nitrosylation of Gpc-1.

To demonstrate that interaction between PrPC and Gpc-1 is a prerequisite for HS degradation, we exposed a mixture of Cu(II)-loaded PrPC and purified Gpc-1 substituted with GlcNH3+-rich HS chains to NO donor and ascorbate. In a major portion of the Gpc-1 molecules, extensive autodegradation of the HS chains was induced (Fig. 7A, filled symbols). Contact between copper-loaded PrPC and the HS chains of Gpc-1 should be necessary for transfer of copper ions, as it is known that interaction with HS weakens Cu(II) binding to PrPC (12–15). In keeping with this proposal, the addition of an excess HS chains inhibited contact between PrPC and Gpc-1 and precluded cleavage of HS (Fig. 7A, open symbols).

It was also of interest to examine whether Zn(II) ions could be transferred from PrPC. However, PrPC preloaded with Zn(II) ions could not support HS degradation (Fig. 7B).

We also tested whether metal ion-loaded Aβ could substitute for PrPC. Aβ participated in the autocleavage reaction in the same way as PrPC, i.e. the Cu(II)-loaded Aβ could support the reaction, but Zn(II)-loaded Aβ could not (Fig. 7, C and D).
average. Given that there are three HS chains/Gpc-1 molecule, a total of 12 SNOs should be required. As there are 14 conserved Cys residues in Gpc-1, nearly all of them could be involved in the cell-free, NO-dependent deaminative autocleavage of Gpc-1 HS. In the cell, however, there could be more extensive degradation of HS, as a continuous supply of NO would rechallenge the Gpc-1 molecules with SNOs.

Apparently, Gpc-1 and PrP⁰ do not coexist at the cell surface of T24 cells (Fig. 6A). However, caveolin-1 was abundant at the cell surface, presumably in caveolae (Fig. 6B). Several studies have shown that caveolae can mediate the uptake of molecules by the budding of transport vesicles from caveolae. These vesicles deliver their cargo to centrally located, caveolin-1-containing structures called caveosomes (for review, see Ref. 35). Structures defined as transporting and sorting endosomes through their involvement in fluid-phase, non-clathrin-dependent endocytosis contain Rab9 as well as caveolin-1 and can transport cargo from the plasma membrane to the Golgi (36, 37). Recent studies have shown that these endosomes (also called caveicles) move along microtubules between caveolae and caveosomes (38). The presence of PrP⁰ in caveolae and interactions between PrP⁰ and caveolin-1 have been reported earlier (6, 22, 39). Binding of prion proteins to the HS chains of recycling Gpc-1 (24–28) could contribute to the observed transfer of scrapie prion infectivity by cell-to-cell contact (40).

The amyloid precursor protein has a classical membrane anchorage, i.e., a membrane-penetrating peptide segment (23). Therefore, it is not expected to be present in rafts or caveolae. However, transmembrane proteins can occur within the same membrane domains as GPI-linked proteins. For example, the major histocompatibility complex type I protein is involved in caveolar uptake of SV40 virus (35) and clustering of transmembrane syndecan-4 (an HS PG) by HS-binding growth factors induces redistribution into raft membrane domains (41). Moreover, a splice variant of the amyloid precursor protein is expressed as a PG called appican (Ref. 42). As discussed previously (28), PG side chains, especially HS, can self-associate and thereby induce clustering.

**The Difference Between Cu(II)- and Zn(II)-supported HS Degradation**—Most of the intracellular Cu(II) ions are bound to proteins (43). Therefore, Cu(II)-mediated degradation of HS in Gpc-1 should involve protein-bound copper ions, either bound to the Gpc-1 molecule itself or to an extrinsic cuproprotein, such as the amyloid precursor protein, or delivered by one, such as PrP⁰. To support S-nitrosylation Cu(II) must be reduced to Cu(I). This can take place either on Gpc-1 or on the amyloid precursor protein but probably not on PrP⁰, at least not normally. Another potential copper donor with potential redox function is the GPI-linked splice variant of ceruloplasmin (44). Although the copper ions needed for Gpc-1 S-nitrosylation/denitrosylation are not consumed, but cycle between Cu(II) and Cu(I), there could be losses that have to be replenished by a copper donor.

Zn(II) ions can also bind to PrP⁰ or to Aβ and probably also to Gpc-1. However, Zn(II) ions do not appear to be transferred from PrP⁰ or Aβ to Gpc-1. Therefore, free Zn(II) ions are probably involved and we propose the mechanism shown in Fig. 9. When Gpc-1 is exposed to Zn(II), Zn-thiolate complexes are formed (Fig. 9, left to middle). When NO donor and ascorbate are provided, Gpc-1 releases Zn(II) ion, the thiolates are oxidized to disulfides, and nitrite and NO are formed (Fig. 9, right). The ensuing deaminative cleavage of the HS chains in the same molecule generates HS fragments (broken lines) and Gpc-1 with truncated HS chains. Zn(II) ions thus support HS degradation by concentrating reactive NO species close to their targets. The formation of disulfides (Fig. 9, right) should be required for the generation of NO⁺. This model is adapted from a study of the transnitrosation of metallothionein III involving NO and transient formation of Zn–thiolate bonds and SNOs (45).

The level of free Zn(II) ions in cells and tissues can vary considerably (7). Gpc-1 is common in the brain, particularly at synapses (46), where the concentration of Zn(II) ions can be especially high (7). It is therefore intriguing that autocatalyzed degradation of HS in Gpc-1 can be supported by free Zn(II) ions. If these are bound to thiolate in Gpc-1, reduction of disulfide bonds would generate more Zn(II)-binding sites. However, reduction did not stimulate autodegradation of HS (Fig. 4D) as in the case of Cu(II) supported degradation (Fig. 2C). It is possible that Zn(II) ions only bind to thiolate present in the native conformation. According to the proposed mechanism for Zn-mediated transnitrosation of metallothionein III (45), SNOs should be transient. Thus, when NO reacts with Zn(II)-loaded Gpc-1, Zn(II) ions are released and a Gpc-1 intermediate containing both SNO and thiolate (S') should be formed. When S' then attacks a neighboring SNO, NO⁺ is formed and the thiolate is oxidized to disulfides. Ascorbate could be involved in this step. As Zn(II) is redox inert, the reaction should stop when all thiols are oxidized, unless a reducing agent is available. This could
not be ascorbate, as ascorbate is unable to reduce disulfide bonds (27, 28). In contrast, the Cu(II)-supported autocleavage of HS should not be conformation-sensitive and could go on indefinitely as long as NO and ascorbate are provided, because the Cu(I) formed in conjunction with SNO formation is reoxidized to Cu(II) when NO is formed.

S-Nitrosylation and the NO Release Mechanism—In cells, NO is generated by the NO synthase-catalyzed conversion of Arg to citrulline and NO. All three NO species can be formed. The proportions depend both on the redox state of the environment and which substrate is available for SNO formation (for review, see Ref. 33). In the cell-free experiments with purified Gpc-1 described here, SNP was used as the NO donor. As shown in this study, NO (II) ions can inhibit Cu(I)-supported autocleavage, which would also result in the generation of HS fragments containing GlcNH3 residues. Other studies have shown that Zn(II) ions can protect a protein against nitrosation of cysteine residues (57). Indeed, reduction in β-amyloid deposition has been achieved by using NO-releasing drugs in an animal model of Alzheimer’s disease (58). It is also intriguing that the capacity for NO production is severely depressed in scrapie-infected cells (59). Our results may provide an explanation for these observations and form the basis of future therapeutic applications.

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