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S-Nitrosylation of secreted recombinant human glypican-1

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Abstract Glypican-1 is a glycosylphosphatidylinositol anchored cell surface S-nitrosylated heparan sulfate proteoglycan that is processed by nitric oxide dependent degradation of its side chains. Cell surface-bound glypican-1 becomes internalized and recycles via endosomes, where the heparan sulphate chains undergo nitric oxide and copper dependent autocleavage at N-unsubstituted glucosamines, back to the Golgi. It is not known if the S-nitrosylation occurs during biosynthesis or recycling of the protein. Here we have generated a recombinant human glypican-1 lacking the glycosylphosphatidylinositol-anchor. We find that this protein is directly secreted into the culture medium both as core protein and proteoglycan form and is not subjected to internalization and further modifications during recycling. By using SDS-PAGE, Western blotting and radiolabeling experiments we show that the glypican-1 can be S-nitrosylated. We have measured the level of S-nitrosylation in the glypican-1 core protein by biotin switch assay and find that the core protein can be S-nitrosylated in the presence of copper II ions and NO donor. Furthermore the glypican-1 proteoglycan produced in the presence of polyamine synthesis inhibitor, α-difluoromethylornithine, was endogenously S-nitrosylated and release of nitric oxide induced deaminative autocleavage of the HS side chains of glypican-1. We also show that the N-unsubstituted glucosamine residues are formed during biosynthesis of glypican-1 and that the content increased upon inhibition of polyamine synthesis. It cannot be excluded that endogenous glypican-1 can become further S-nitrosylated during recycling.

Keywords Ascorbate · Proteoglycans · Heparan sulfate · Glycosaminoglycans · Nitric oxide · Difluoromethylornithine · Nitrosothiols

Abbreviations

DFMO	α-Difluoromethylornithine
GAG	Glycosaminoglycans
$GlcNH_3^+$	N-unsubstituted glucosamine
Gpc-1	Glypican-1
GPI	Glycosylphosphatidylinositol
HS	Heparan sulfate
mAb	Monoclonal antibody
NO	Nitric oxide
PG	Proteoglycan
SNO	Nitrosothiol
SNP	Sodium nitroprusside dihydrate

Introduction

Glypicans constitute a family of glycosylphosphatidylinositol (GPI)-anchored, lipid-raft-associated, cell-surface heparan sulfate proteoglycans (HSPG). They are involved in selective regulation of growth factor and cytokine signaling and in the uptake of biomolecules including polyamines, peptides, nucleic acids and viruses [1, 2]. To date, six different glypicans have been identified in mammals, two in *Drosophila Melanogaster*, one in *Caenorhabditis elegans*, and one in zebrafish [3, 4]. The core proteins of glypicans contain an N-terminal signal peptide, a globular domain

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containing 14 conserved Cys residues, followed by a GAG attachment domain that includes multiple Ser-Gly dipeptide sites and a C-terminal signal peptide responsible for glycosylphosphatidylinositol (GPI)-anchorage of the core protein to the plasma membrane.

Thiols can be substituted with nitric oxide (NO) forming nitrosothiols (SNO). Many proteins have been found to be S-nitrosylated. Protein S-nitrosylation is an important dynamic modification of proteins that affect numerous cellular processes [5]. Cys residues flanked by acidic/basic amino acid residues in the primary sequence of proteins are considered to be preferred targets for S-nitrosylation [6]. However, such arrangements can also arise from the three-dimensional protein structure. In the primary sequence of Gpc-1 only a minority of the Cys residues are in acidic/basic motifs. We have previously demonstrated that the PG form of glypican-1 (Gpc-1) can be S-nitrosylated on Cys residues in the core protein forming Gpc-1-SNO [7]. The reaction is copper dependent and cuproteins such as amyloid precursor protein (APP), prion protein and the brain-specific GPI-linked splice variant of ceruloplasmin can deliver the Cu(II) ions required for S-nitrosylation of Gpc-1 *in vitro* as well as *in vivo* [8, 9]. Gpc-1-SNO recycles from the cell surface probably via a caveolar endocytosis to early and late endosomes and then back to the Golgi. In late endosomes a reducing agent releases NO, which results in deaminative cleavage of the HS chains at N-unsubstituted glucosamines ($GlcNH_3^+$) [9–13]. Thus, factors in the Gpc-1 core protein affect the HS chains and modulate its biological function. The HS chains of Gpc-1 PG usually contain a small amount of $GlcNH_3^+$ near the core protein attachment [14, 15]. However, the number of $GlcNH_3^+$ residues increases markedly upon inhibition of polyamine synthesis with α-difluoromethylornithine (DFMO) [14, 16]. It is unclear whether S-nitrosylation of Gpc-1 and formation of $GlcNH_3^+$ takes place during biosynthesis, maybe prior to HS attachment, or during recycling. Here we have generated a recombinant human Gpc-1 (rhGpc-1) lacking the GPI-anchor that is secreted into the culture medium. Both HS substituted and unsubstituted Gpc-1 were generated. By adapting a method based on biotin labeling of S-nitrosylated cysteines we demonstrate S-nitrosylation of copper II and NO donor treated core protein in the absence of HS substitution. We also analyzed S-nitrosylation of rhGpc-1 PG and the content of $GlcNH_3^+$ in HS chains in polyamine synthesis inhibited cells by monitoring auto-cleavage of the HS side chains by gel permeation chromatography or SDS-PAGE. We find the rhGpc-1 produced in the presence of the polyamine synthesis inhibitor, DFMO, became S-nitrosylated and the amount of $GlcNH_3^+$ residues was markedly increased indicating that S-nitrosylation and generation of $GlcNH_3^+$ take place during biosynthesis.

Materials and methods

Materials

The plasmid pCEP4-BM40-HisEK [17] derived from the Invitrogen plasmid pCEP4 and EBNA 293 cells were kind gifts from Dr. Ahnders Franzén, Lund University. HEK 293 cell line was a kind gift from Professor Anders Malmström, Lund University. Human Gpc-1 cDNA clone, IMAGE ID 6275649, was obtained from Geneservice (UK). The restriction enzymes BamHI and HindIII were from MBI Fermentas GmbH (Germany). The Expand High Fidelity PCR system was obtained from Roche (Germany). T4 DNA ligase, Lipofectamine, fetal bovine serum (FBS), minimal essential medium with Earle's salts (MEM), 4–12% Bis-Tris and 3–8% Tris-Acetate SDS-PAGE gels, colloidal Coomassie Blue staining kit were obtained from Invitrogen (Carlsbad, CA). Ni-NTA columns, EX-CELL 325 PF CHO serum-free medium, monoclonal anti-biotin clone BN-34, DFMO and fluorometric nitric oxide synthase detection system were from Sigma-Aldrich (St. Loius, MO). 1-Biotinamido-4-(4′-[maleimidomethyl-cyclohexane]-carboxyamido)butane (Biotin-BMCC) was from Pierce Biotechnology (Rockford, IL). *N*-Ethylmaleimide (NEM) was from Boehringer-Ingelheim (Germany). D-[6-^{3}H]-glucosamine (40 Ci/mmol) was purchased from Amersham International (UK). Prepacked column of Superose 6 HR 10/30 was from Pharmacia-LKB (Sweden) and DE-53 DEAE was from Whatman (UK). Micro Bio-Spin 6 chromatography columns were from Bio-Rad Laboratories (Hercules, CA). The monoclonal antibody 10E4 and antiserum to human Gpc-1 were obtained as described previously [18].

Plasmid construction

Human Gpc-1 cDNA was amplified by PCR using the forward primer 5′-TT**AAGCTT**GACCCGGCCA GCAAGAG-3′ and the reverse primer 5′-TT**GGAT CC**TTAGGTCTTCTGTCCTTCCTGCTC-3′ creating a DNA fragment comprising Gpc-1 cDNA base pairs 70–1587 coding sequence of human glypican-1 mRNA with NCBI nucleotide database (GenBank) accession number BC051279. Restriction sites for cloning were inserted at the 5′ end of the primers and are marked in boldface. The PCR product was digested with the restriction enzymes *BamHI* and *HindIII* and inserted into the *BamHI*/*HindIII* digested plasmid pCEP4-BM40-HisEk. The plasmid contains a sequence coding for a BM40 signal peptide for efficient extracellular transport of recombinant protein. It also contains sequences coding for a hexahistidine region and an enterokinase cleavage site. Plasmid containing the correct insert was analyzed by agarose gel after digestion

of purified plasmid with *HindIII* and *BamHI*, and the introduced sequence was confirmed by DNA sequencing at MWG Biotech (Germany).

Cell culture and extraction

EBNA 293 and HEK 293 cells were cultured as monolayers in MEM supplemented with 10% (*v*/*v*) FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) in an incubator with humidified atmosphere and 5% CO_2 at 37°C, unless otherwise stated. Cells were extracted with 0.1–0.2 ml/cm^2 dish of 0.15 M NaCl, 10 mM EDTA, 2% (*v*/*v*) Triton X-100, 10 mM KH_2PO_4, pH 7.5, 5 μg/ml ovalbumin containing 1 mM diisopropylphosphorofluoridate on a slow shaker at 4°C for 10 min.

Transfection

Cells were transfected with pCEP4-BM40-HisEK-Gpc-1. Transfection was performed using Invitrogen's standard protocol for transfection with Lipofectamine. Sixty thousand cells were seeded into the wells of a 6-well plate and grown for 24 h to 50% confluence. Plasmid and Lipofectamine were mixed in MEM without FBS and incubated for 45 min at room temperature. The cells were incubated with 0.2 ml of medium containing plasmid and Lipofectamine for 5 h in 37°C and then 0.4 ml MEM supplemented with 20% (*v*/*v*) FBS was added. Three days after the transfection, the cells were split 1:10 into MEM supplemented with 10% (*v*/*v*) FBS, 2 mM L-glutamine and 200 μg/ml hygromycin B. After several weeks of growth in the selective medium, the cells were cloned by limiting dilution. The clones were analyzed for expression of rhGpc-1. Clones expressing a large amount of rhGpc-1 were expanded and used in this study.

Anion exchange chromatography isolation of radiolabeled rhGpc-1 PG

Cells with high expression of rhGpc-1 were grown to confluence and pre-incubated for 1 h in low-sulfate, $MgCl_2$-containing medium supplemented with 2 mM glutamine in the case of [^{35}S]-sulfate radiolabeling. The pre-incubation medium was replaced by fresh medium containing 20 mCi/ml of [^{3}H]-glucosamine or 50 μCi/ml [^{35}S]-sulfate with or without 5 mM DFMO. After the incubation period of 48 h, culture medium was collected and pooled with two washings of ice-cold PBS (0.137 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5). The conditioned radiolabeled medium was applied to a 1 ml-column of DEAE-cellulose equilibrated with 6 M urea, 0.5 M NaOAc, pH 5.8, 5 μg/ml ovalbumin, 0.1% Triton X-100. After sample application, the columns were washed successively with 10 ml-portions of a) equilibration buffer (see above), b) 6 M urea, 10 mM Tris-HCl, pH 8.0, 5 μg/ml ovalbumin, 0.1% Triton X-100 and c) 50 mM Tris-HCl pH 7.5. Bound material was eluted with 5×1 ml 4 M guanidine-HCl, 50 mM NaOAc, pH 5.8, 5 μg/ml ovalbumin, 0.2% Triton X-100 and the radioactivity was determined in a β-counter. Fractions containing radioactivity were then pooled, and precipitated with 5 volume of 95% ethanol overnight at −20°C using 100 μg of dextran as carrier. After centrifugation in a Beckman JS-7.5 at 3000× g at 4°C for 45 min the material was dissolved in 4 M guanidine-HCl, 50 mM NaOAc, pH 5.8 and subjected to gel permeation chromatography on Superose 6.

In order to isolate native form of rhGpc-1 PG [^{3}H]-glucosamine labeled polyanionicmacromolecules were isolated from the culture medium (EX-CELL 325 PF CHO Serum-Free Medium) supplemented with 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) by ion-exchange chromatography on DEAE-cellulose at 4°C. Conditioned medium was passed over 1 ml-columns of DEAE-cellulose equilibrated with PBS. After sample application, the columns were washed successively with 10 ml-portions of PBS × 5. Bound material was eluted with 2×1 ml 0.5 M NaCl followed by 2×1 ml 1.0 M NaCl and the radioactivity was determined in a β-counter.

Ni-NTA and anion exchange chromatography purification of rhGpc-1 and Western blot analysis

Two cell clones with high expression of rhGpc-1 were grown separately to confluence in MEM supplemented with 10% (*v*/*v*) FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 200 μg/ml hygromycin B. After extensive washing with PBS, the medium was replaced with EX-CELL 325 PF CHO Serum-Free Medium supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 100 μg/ml hygromycin B. The conditioned medium was harvested after 3 or 4 days and dialyzed against 0.3 M NaCl, 50 mM Na-phosphate, pH 8.0. Approximately 100 ml of dialyzed conditioned medium was applied to a Ni-NTA column according to the instructions of the manufacturer. After extensive washing, the bound rhGpc-1 was eluted in a single peak using a linear imidazole gradient. In order to separate the PG form of Gpc-1 from non-glycosylated core protein the material in the washings and in the imidazole eluate from the Ni-NTA columns were diluted two-times with water and then subjected to anion exchange chromatography on DEAE-cellulose columns. The bound PG was eluted using 1 M NaCl and the purity was analyzed on a 4–12% Bis-Tris SDS-page gel followed colloidal Coomassie Blue staining according to the protocol of the manufacturer. For Western

blot analysis the samples were subjected to SDS-PAGE on 4–12% Bis-Tris gels and then transferred to polyvinylidine difluride (PVDF) membranes. After blocking with 10% non-fat milk, 0.05% Tween 20 in PBS, membranes were incubated with either anti-human-Gpc-1 polyclonal antibody (1:5000) or monoclonal antibody 10E4 (1:1000) overnight at 4°C. After washing, the membranes were probed with suitable horse-radish peroxidase-conjugated antibodies (1:5000) for 1 h at room temperature and developed using a Fujifilm ECL detector.

Detection of S-nitrosylation

S-Nitrosylation was detected by adapting the method of Jaffrey *et al*. [19]. The experiment was performed in the dark until the Western blot analysis. 1:10 volume of 1 mM sodium nitroprusside dihydrate (SNP) in 0.5 mM $CuCl_2$ was added to 60 μg of Gpc-1 in 100 μl PBS with 0.1 mM neocuproine, pH 7.2 and incubated for 20 min at 20°C to S-nitrosylate Gpc-1. SNP was then removed by using a Micro Bio-Spin P6 column according the instructions of the manufacturer. Four volumes of blocking buffer (20 mM NEM in 9 volumes of PBS with 0.1 mM neocuproine, pH 7.2 and 1 volume of 25% *w/v* SDS in millipore water, prepared fresh) was added to the Micro Bio-Spin P6 elutes and the samples were incubated for another 20 min at 50°C in order to block the unbound thiol groups, which would become available when the protein was denatured by SDS. The NEM was then removed by acetone precipitation of the protein and the samples were dissolved in 100 μl PBS with 0.1 mM neocuproine, pH 7.2. The labeling buffer (8.5 mM biotin-BMCC in 0.55 ml DMSO) and ascorbate solution (50 mM in water) were prepared immediately before use. 1:3 volume of labeling buffer and 1:50 volume of ascorbate solution were added to the samples and incubated for 1 h at room temperature in order to biotinylate recombinant Gpc-1 at the potential S-nitrosylation sites. The samples were then analyzed by Western blot using anti-biotin mouse monoclonal antibody (1:500). The PVDF membrane was stripped and re-probed with anti-human-Gpc-1 polyclonal antibody (1:5000).

Autocleavage of HS in native rhGpc-1 PG and analysis with SDS-page or gel chromatography

Conditioned medium from Gpc-1 transfected cells cultured treated with 5 mM DFMO was used in these experiments. rhGpc-1 PG was purified by anion exchange chromatography on the material obtained by Ni-NTA purification as described above. Different combinations of 3 mM SNP, 20 μM $CuCl_2$ and 6 mM ascorbate were added to 15 μg of Gpc-1 PG in 100 μl of PBS. When both SNP and ascorbate were added to the samples, SNP was added first and the samples were incubated for 1 h at 37°C in the dark. After addition of ascorbate, the samples were incubated overnight at 37°C. Two μg of treated Gpc-1 PG were loaded per well on 3–8% Tris-Acetate SDS-page gels. The gels were stained with colloidal Coomassie Blue according to the protocol of the manufacturer.

For determination of autodegradation in [^{3}H]-glucosamine labeled rhGpc-1 produced by DFMO-treated cells, the native rhGpc-1 PG isolated by anion exchange chromatography without urea was exposed to different combinations of 1 mM SNP, 10 μM $CuCl_2$, and 1 mM ascorbate in PBS, pH 7.4, at 37°C. The reactions were monitored by Superose 6 gel chromatography [7].

Degradation procedures

GAG chains were released from PG by treatment with 0.5 M NaOH, 0.1 M $NaBH_4$ at room temperature overnight. Samples were neutralized with HOAc, and then subjected to gel permeation chromatography on Superose 6 as described elsewhere [11]. HS chains were degraded by deaminative cleavage by using HNO_2 at pH 1.5 [20]. Cleavage of HS chains at $GlcNH_3^+$ was carried out with HNO_2 at pH 3.9 [21]. In both cases samples were subjected to gel permeation chromatography on Superose 6 and the radioactivity was determined in a β-counter.

Results

Generation of a vector encoding rhGpc-1

A cDNA for human Gpc-1 (GenBank accession number NN_002081) was introduced in the mammalian expression vector pCEP4-BM40-HisEk. The construct lacked the endogenous sequence coding for the N-terminal signal peptide responsible for translocation to ER, and the sequence coding for the C-terminal signal peptide responsible for GPI-attachment to the membrane. Instead the vector encoded Gpc-1 with an N-terminal BM40 secretion signal peptide, a His_6 tag and an enterokinase cleavage site. We had thereby created a vector encoding a His_6-tagged Gpc-1, which should become secreted into the culture medium.

Expression, purification and characterization of rhGpc-1

EBNA 293 cells which produce NO (see Supplemental data, Fig. S1) were transfected with the human Gpc-1-vector and then cultured under the selective pressure of hygromycin B in order to maintain a stable cell line expressing rhGpc-1. qRT-PCR and Western blot analysis showed that the cells had become transfected and that the

rhGpc-1 was secreted to the culture medium (data not shown). Cells were cloned by limiting dilution and clones with high expression were selected for use in further studies. Approximately 100 ml of conditioned medium was applied to a Ni-NTA column and a single peak of protein was eluted by a linear imidazole gradient (Fig. 1A). The protein was analyzed on SDS-PAGE stained with colloidal Coomassie Blue and yielded a band of ~70 kDa and a smear of ~70–170 kDa (Fig. 1B). Approximately 3 mg protein was obtained from 100 ml of conditioned medium. The single band and the protein smear were separated by anion exchange chromatography. MALDI-TOF experiments identified the ~70 kDa band as Gpc-1 with sequence coverage of 37% (data not shown). Both the rhGpc-1 band and the smear reacted with an antibody against Gpc-1 (Fig. 1C). Thus, we had expression of both the core protein and the PG form of Gpc-1. The expression of rhGpc-1 with heavily reduced GAG substitution was probably due to the over-expression and has been observed when over expressing other PGs [22].

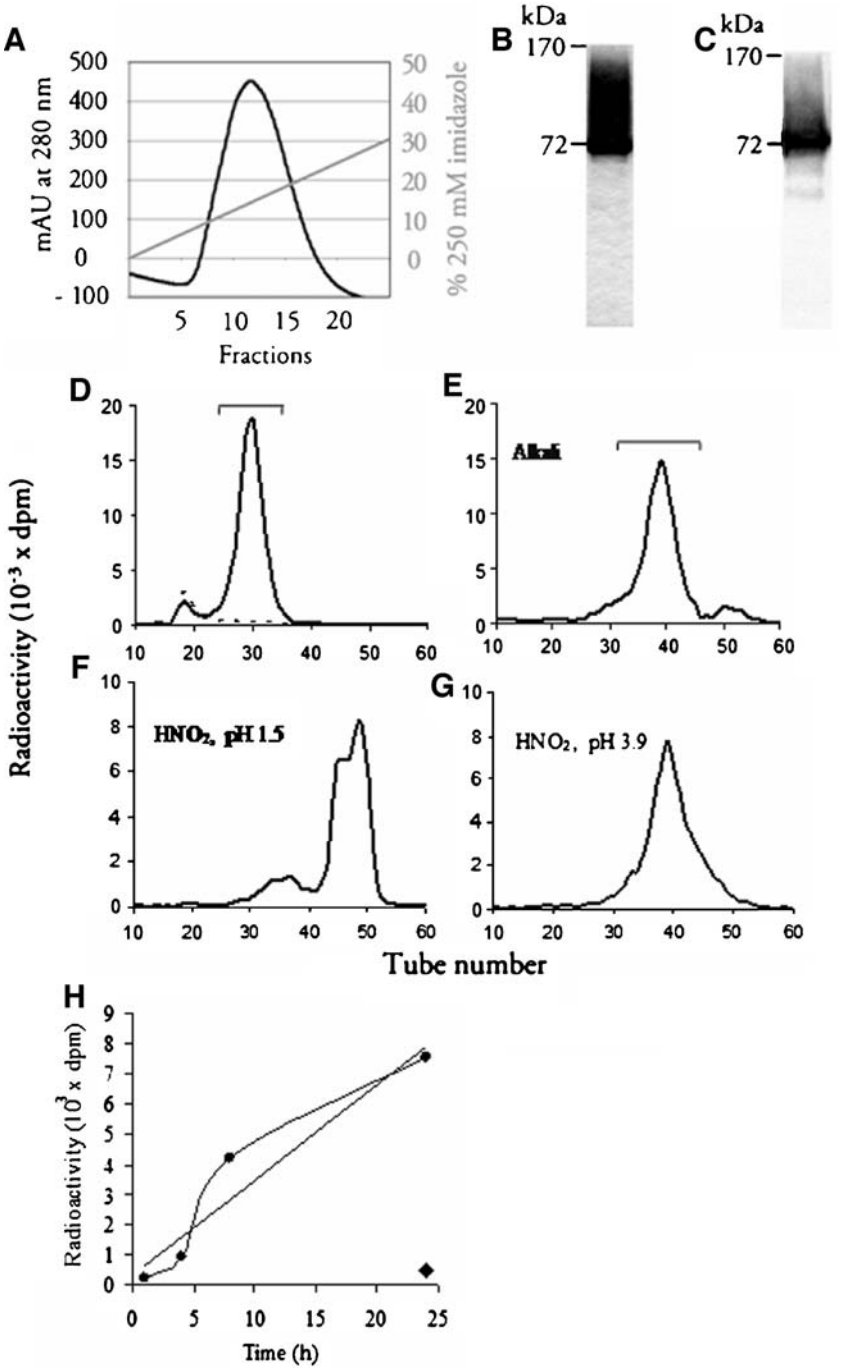

Fig. 1 Purification and characterization of rhGpc-1. **A** Conditioned medium from EBNA 293 cells transfected with pCEP4-BM40-HisEK-GPC-1 was applied to a Ni-NTA column. After extensive washing a single peak was eluted using a linear imidazole gradient. **B** Pooled material was analyzed on SDS-PAGE stained with colloidal Coomassie Blue or **C** was subjected to Western blotting using antibodies against Gpc-1. **D** Gel permeation chromatography of [^{3}H]-glucosamine labeled polyanionic material secreted from rhGpc-1 transfected EBNA 293 cells and isolated with anion exchange chromatography. **E** Pooled material in **D** (see *bar*) after alkaline borohydride treatment. **F** Pooled material in **E** (see *bar*) after treatment with HNO_2 at pH 1.5 or **G** after treatment with HNO_2 at pH 3.9. **H** rhGpc-1 transfected EBNA 293 cells were incubated with [^{35}S]-sulfate and the amount of radiolabeled material isolated with anion exchange chromatography was analyzed after different periods of time. The ♦ symbol in Fig. 4H shows the amount of internalized material after 24 h incubation

To characterize the structure of the GAG chains attached to the core protein, metabolic radiolabeling with [^{35}S]-sulfate and [^{3}H]-glucosamine was performed in untransfected and Gpc-1 transfected EBNA 293 cells. Radiolabeled PG was isolated from conditioned medium by Ni- NTA and anion exchange purification or by only anion exchange purification followed by gel permeation chromatography on Superose 6. In Gpc-1 transfected cells both purification methods showed the same profile on Superose 6 indicating that Ni-NTA purification was not required for isolation of radiolabeled material (Fig. 1D). Both [^{35}S]-sulfate and [^{3}H]-glucosamine labeling followed the same profile on Superose 6. We therefore used only [^{3}H]-glucosamine labeling and anion exchange chromatography for further characterization of the GAG chains. The [^{3}H]-glucosamine labeled material from non-transfected cells consisted of a minor pool that was eluted at the void of Superose 6 (Fig. 1D, dashed line). Gpc-1 transfected cells secreted radiolabeled material to the cultured medium that was more retarded on Superose 6 (Fig. 1D, solid line). This material was sensitive to alkali (Fig. 1E) and the chain size of the major pool (see bar in Fig. 1E) was estimated to be 30–50 kDa (fractions 30–43) indicating that mature sized GAG chains were assembled on the core protein. Some smaller chain stubs were also present (fractions 45–55). The alkali-released GAG chains were not sensitive to chondroitin ABC lyase treatment but were subsequently cleaved by HNO_2 treatment at pH 1.5 (Fig. 1F) indicating that they consisted of HS chains. To estimate the content of $GlcNH_3^+$, the alkali-released HS-chains were treated with HNO_2 at pH 3.9 and rechromatographed. A small shift in the profile was detected (Fig. 1G) and the material consisted of HS fragments with an estimated average size of 20–50 kDa (fractions 30–50). The marginal effect of HNO_2 pH 3.9 on the HS side chains of rhGpc-1 may be due to low content of $GlcNH_3^+$ residues. However, localization of clusters of $GlcNH_3^+$ residues located near the non-reducing terminus of the released HS fragments could not be excluded. In this case, a small size-change caused by cleavage with HNO_2 would be difficult to detect with this approach.

To further investigate the secretion and uptake of rhGpc-1, the cells were incubated with [^{35}S]-sulfate for increasing periods of time and polyanionic macromolecules secreted into the culture medium were isolated. Accumulation of radiolabeled polyanionic material in the culture medium with time was observed (Fig. 1H). At the time point of 24 h 93% of polyanionic radiolabeled material was secreted in the culture medium and 7% was detected in the cell extracts. Furthermore, when the isolated radiolabeled material was added to a culture of non-radioactive cells ~5% uptake was detected (Fig. 1H, ♦ symbol). Taken together these results indicate secretion of anchorless rhGpc-1 to the culture medium.

S-Nitrosylation of rhGpc-1

Previous results from this laboratory using indirect methods such as immunostaining with antibodies against SNO or autocleavage of HS chains upon treatment with NO-releasing agents indicated that endogenous recycling Gpc-1 can become S-nitrosylated. In order to investigate S-nitrosylation of rhGpc-1 and to explore whether S-nitrosylation takes place during biosynthesis or recycling of Gpc-1 the present study was undertaken. S-Nitrosylation of Ni-NTA and anion exchange chromatography purified core protein was investigated by adapting the method of Jaffrey and Snyder [19] based on biotin labeling of previously S-nitrosylated Cys residues. The biotin switch assay was analysed by Western blot using biotin antibody. A weak biotin positive band for

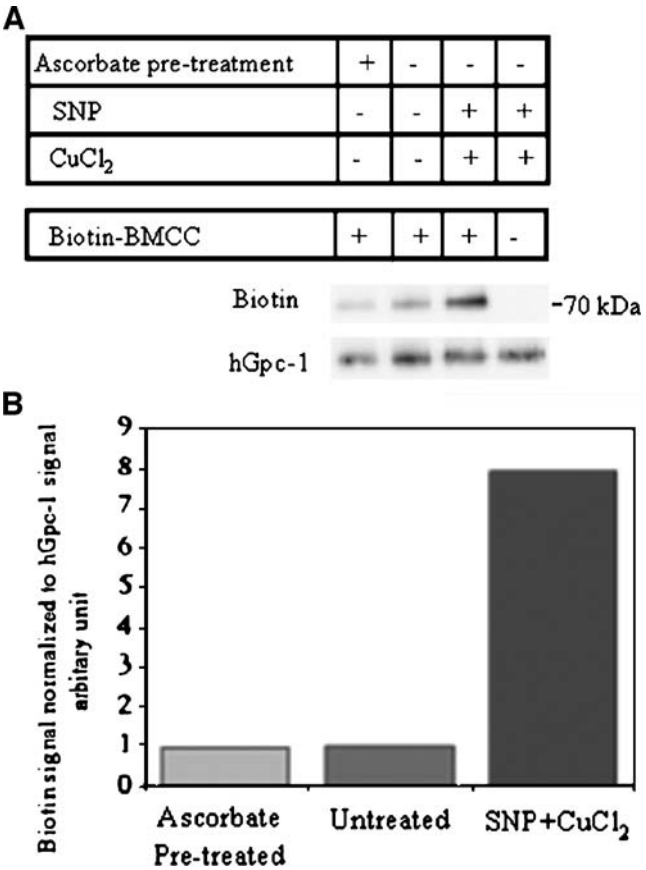

Fig. 2 Detection of S-nitrosylation in rhGpc-1 core protein using biotin switch assay. 60 μg of rhGpc-1 core protein was pre-incubated with 10 mM ascorbate overnight or incubated with 0.1 mM SNP and 50 μM $CuCl_2$ for 20 min as indicated in the figure. The samples were then subjected to biotin switch assay as described in Materials and methods. **A** Biotinylated S-nitrosylation sites were detected with Western blot using anti-biotin. Stripped membrane was reprobed with anti-Gpc-1 to estimate the amount of Gpc-1 loaded in the wells. The results have been confirmed twice. **B** The band intensities were quantified using Gel-Pro Analyzer software and normalized for Gpc-1 signal in the untreated sample

rhGpc-1 core protein appeared at ~70 kDa in untreated samples (Fig. 2A). Ascorbate pre-treatment of rhGpc-1, which should release all NO from rhGpc-1 was used as a control. The weak signal observed when rhGpc-1 was pre-treated with ascorbate suggested an insufficient blocking of thiols, which could later be labeled by biotin-BMCC giving rise to some background signal. A stronger signal was detected when rhGpc-1 was pre-treated with NO donor and copper ions. As a negative control the labeling substance, biotin-BMCC, was omitted from one of the samples without detecting any signal. The western blot membrane was further stripped and reprobed with anti-Gpc-1 to estimate the amounts of protein that had been loaded in the wells. The intensities of the bands were quantified using Gel-Pro Analyzer and the data were normalized relative to the anti-Gpc-1 signal of the untreated sample (Fig. 2B). According to this analysis, an eightfold increase of signal intensity was measured when rhGpc-1 had been exposed to NO-donor and copper ions. Based on these results it appears that rhGpc-1 core protein contains free thiols that can be S-nitrosylatedin the presence of NO donor and cupric ions.

The effect of inhibition of polyamine synthesis on the HS structure and core protein S-nitrosylation in rhGpc-1 PG

Studies from this laboratory have indicated that inhibition of endogenous polyamine synthesis with DFMO induces an increased number of NO-sensitive $GlcNH_3^+$ residues [14]. The mechanism of $GlcNH_3^+$ formation and whether this occurs during biosynthesis or recycling of Gpc-1 has been unclear. In order to study the effect of inhibition of polyamine synthesis, metabolically radiolabeled rhGpc-1 produced in the presence of DFMO was isolated by anion exchange chromatography. The product was analyzed by gel chromatography before and after alkali treatment and by HNO_2 treatments of released HS at pH 1.5 and 3.9, respectively (Fig. 3). The Gpc-1 glycoforms obtained from DFMO treated cells were the same size as the corresponding material from untreated cells (*cf.* Figs. 3A and 1D). The HS chains derived from these glycoforms had also almost the same size (average size of 40 kDa) as the corresponding material from untreated cells (*cf.* Figs. 3B and 1E). However, inhibition of polyamine synthesis by DFMO induced production of rhGpc-1 with HS chains that were extensively depolymerized upon deaminative cleavage at pH 3.9 to HS fragments with a chain size between 10–30 kDa (fractions 40–52) indicating an increased amount of $GlcNH_3^+$ with uniform distribution in all the HS chains of the rhGpc-1 PG (*cf.* Figs. 3D and 1G).

To investigate S-nitrosylation of rhGpc-1 expressed in the presence of DFMO, Ni-NTA and anion exchange chromatography purified rhGpc-1 core proteins from untreated and DFMO treated cells were subjected to biotin-switch assay. A weak biotin positive band for rhGpc-1 core protein appeared at ~70 kDa in untreated samples, whereas a stronger signal was detected for rhGpc-1 core protein produced in the presence of DFMO (Fig. 4A). The western blot membrane was further stripped and reprobed with anti-Gpc-1 to estimate the amounts of core protein and the intensities of the bands were quantified using

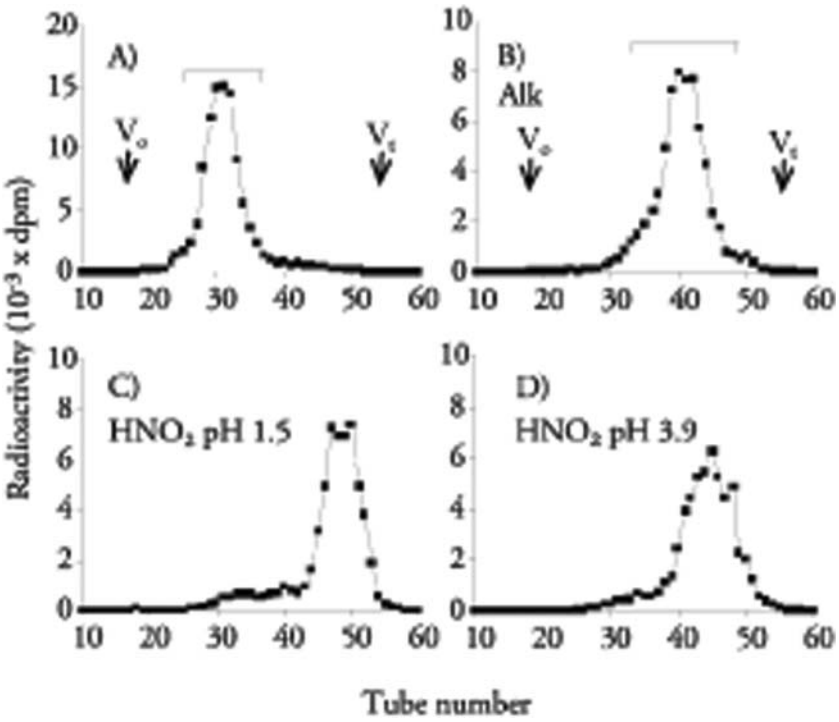

Fig. 3 Effect of polyamine synthesis inhibition on the structure of HS in rhGpc-1 PG. EBNA 293 cells expressing rhGpc-1 were deprived of polyamines by treatment with 5 mM DFMO. **A** [^{3}H]-Glucosamine was added to the cells and radiolabeled poly-anionic material secreted by the cells was isolated by anion exchange chromatography and analyzed by gel permeation chromatography. **B** Pooled material in **A** (see *bar*) after alkaline borohydride treatment. **C** Pooled material in **B** (see *bar*) after treatment with HNO_2 at pH 1.5 or **D** after treatment with HNO_2 at pH 3.9

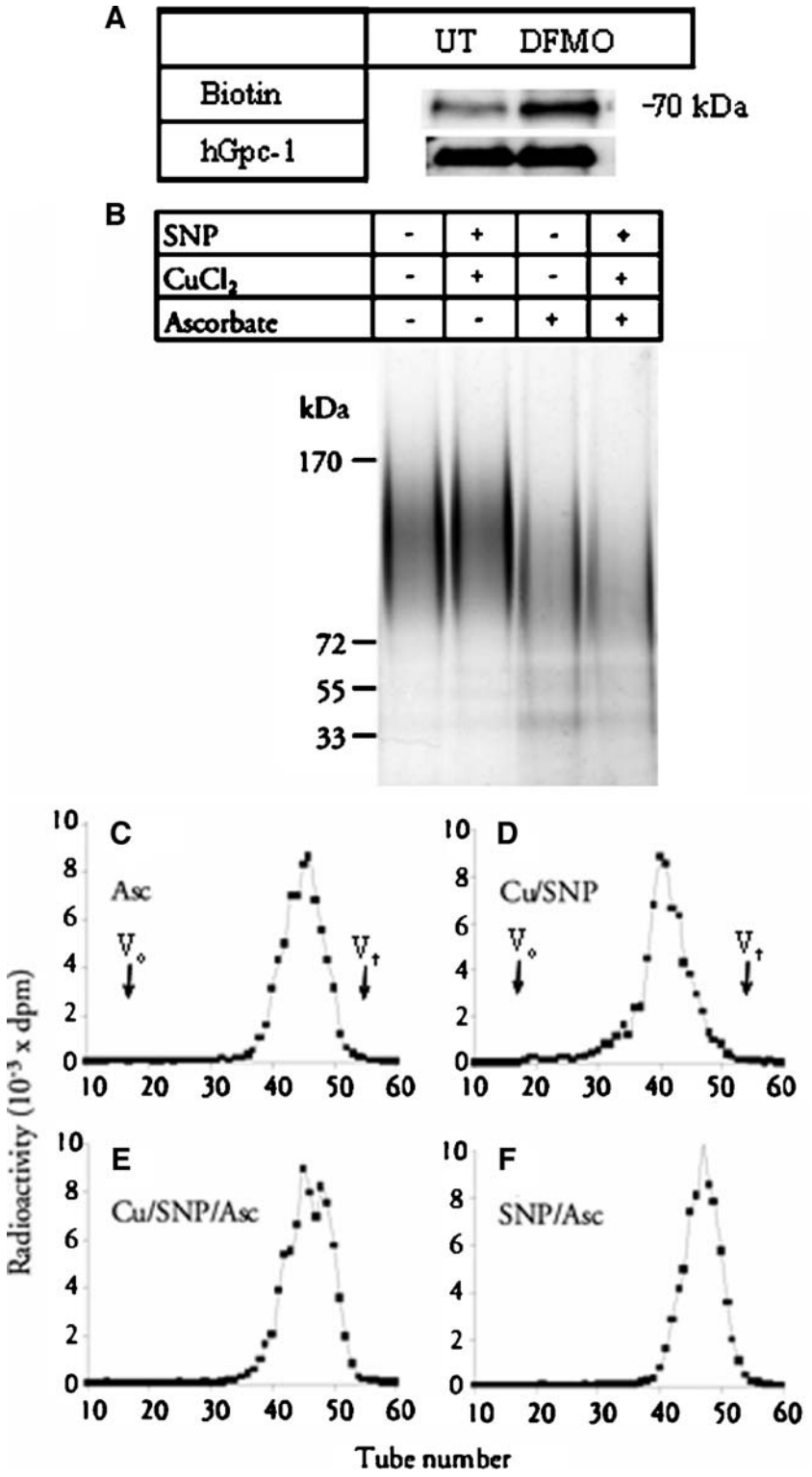

Fig. 4 Effect of DFMO on S-nitrosylation of rhGpc-1. **A** rhGpc-1 core protein isolated by Ni-NTA and ion-exchange chromatography from conditioned medium of transfected cells cultured in the absence or presence of 5 mM DFMO was subjected to biotin switch assay. Biotinylated S-nitrosylation sites were detected with Western blot using anti-biotin. Stripped membrane was reprobed with anti-Gpc-1 to estimate the amount of Gpc-1 loaded in the wells. **B**–**F** rhGpc-1 PG was purified from conditioned medium of transfected cells cultured in the presence of 5 mM DFMO by **B** Ni-NTA and ion-exchange chromatography or **C**–**F** ion exchange chromatography. **B** 15 μg of Gpc-1 PG was incubated overnight with 3 mM SNP, 20 μM $CuCl_2$ and 6 mM ascorbate as indicated in the figure. The samples were run on SDS-PAGE and stained with colloidal Coomassie Blue. **C**–**F** Gel permeation chromatography of [^{3}H]-glucosamine labeled polyanionic material incubated with 1 mM SNP, 10 μM $CuCl_2$ and 1 mM ascorbate as indicated in the figure followed by alkaline borohydride treatment

Gel-Pro Analyzer. The data were normalized relative to the anti-Gpc-1 signal of the untreated sample. According to this analysis, a 3.7 fold increase of signal intensity was measured for rhGpc-1 produced in the presence of DFMO indicating S-nitrosylation. To further investigate S-nitrosylation and autocatalyzed deaminative degradation of HS in rhGpc-1 PG produced by DFMO-treated cells we used both unlabeled and [^{3}H]-glucosamine labeled rhGpc-1 expressed in the presence of DFMO. Ni-NTA and ion exchange chromatography purification were used to isolate the native unlabeled rhGpc-1 and anion exchange chromatography to isolate the native [^{3}H]-glucosamine labeled rhGpc-1. The isolated PGs

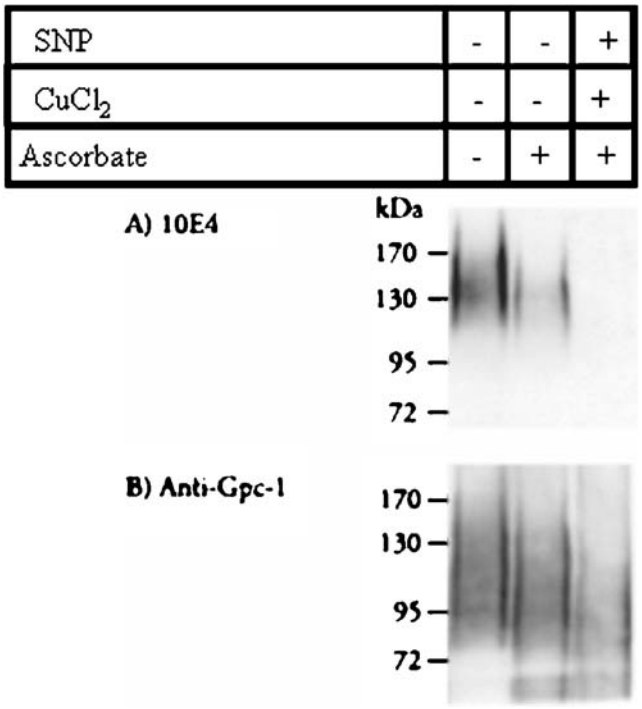

Fig. 5 Detection of $GlcNH_3^+$ containing units in rhGpc-1 PG from DFMO-treated cells using mAb 10E4. rhGpc-1 PG was purified from conditioned medium of cells grown in 5 mM DFMO by Ni-NTA and ion exchange chromatography without urea. 15 μg of Gpc-1 PG was incubated overnight with 3 mM SNP, 20 μM $CuCl_2$ and 6 mM ascorbate as indicated in the figure. The samples were subjected to SDS-PAGE and analyzed by Western blotting using **A** mAb 10E4 which recognizes $GlcNH_3^+$ and **B** then stripped and reprobed with anti-Gpc-1

were then subjected to treatments with different combinations of $CuCl_2$, SNP and ascorbate and the effect was analyzed using SDS-PAGE for the unlabeled rhGpc-1 and gel permeation chromatography of alkali cleaved HS chains on Superose 6 for the [^{3}H]-glucosamine labeled rhGpc-1. Interestingly, ascorbate treatment alone induced depolymerization of HS side chains (Fig. 4B and C). However, some degradation of the rhGpc-1 core protein by ascorbate was also detected. The extensive depolymerization observed in [^{3}H]-glucosamine labeled HS chains was similar to that obtained with HNO_2 at pH 3.9 inducing formation of HS fragments uniform in size with an average size of approximately 20 kDa (fractions 39–52, approximately 10–30 kDa) (*cf.* Figs. 4C and 3D). No HS degradation was detected when the recombinant protein was exposed to only $CuCl_2$ and NO-donor (Fig. 4B and D). However, when ascorbate was included an extensive depolymerization of HS chains took place generating HS fragments with a size varying from 30 down to 10 kDa (Fig. 4B and E). Furthermore, omission of copper did not affect the extent of depolymerization (Fig. 4F). These results suggest that the [^{3}H]-glucosamine labeled Gpc-1 PG secreted by DFMO treated cells contained sufficient SNO to sustain an extensive NO-dependent degradation of HS.

Deaminative cleavage of HS chains at $GlcNH_3^+$

The mAb 10E4 recognizes an epitope containing $GlcNH_3^+$ residues and is commonly used to detect HSPGs. Using confocal microscopy and biochemical assays we previously showed that the 10E4 epitope is sensitive to treatment of HS chains with HNO_2 at pH 3.9 as well as with ascorbate [18]. To further characterize the NO cleavage sites in HS chains of unlabeled rhGpc-1 PG we studied the effect of NO donor, $CuCl_2$, and/or ascorbate on HS degradation using Western blot with the mAb 10E4. The 10E4 epitope was present in higher molecular size variants of rhGpc-1 PG (M_r approx. 100–170 kDa) indicating the presence of $GlcNH_3^+$ moieties preferentially in PG with long side chains (Fig. 5A). When such Gpc-1 was exposed to ascorbate the 10E4 signal diminished markedly and the size of rhGpc-1 PG was reduced. Exposure of rhGpc-1 PG to a combination of SNP, $CuCl_2$ and ascorbate resulted in complete disappearance of the 10E4 signal indicating complete cleavage of the HS chains at the $GlcNH_3^+$ residues. The Western blot membrane was then stripped and reprobed with anti-Gpc-1 to assess the effects on the size of Gpc-1 PG (Fig. 5B). The results showed that the disappearance of the 10E4 signal yielded a corresponding reduction in the size of the PG (Fig. 5B). Some degradation of the rhGpc-1 core protein by ascorbate was also detected (Fig. 5B). Taken together these results demonstrate that the secreted unlabeled rhGpc-1 PG contains sufficient SNO groups to cleave a major portion of the NO-sensitive glucosaminidic bonds next to $GlcNH_3^+$ giving rise to PGs with shorter GAG chains.

Discussion

The present results demonstrate that a) anchorless rhGpc-1 is secreted into the culture medium, b) the secreted rhGpc-1 is both unsubstituted and substituted with HS chains, c) the Gpc-1 glycoforms contain HS chains with the same size as native Gpc-1 produced in other cell types with an estimated size of 30–50 kDa [23], d) the HS chains in the rhGpc-1 PG contain $GlcNH_3^+$ residues clustered near the linkage region, e) secreted non-glycanated rhGpc-1 core protein contains free Cys residues that can become S-nitrosylated by NO donor and $CuCl_2$, f) inhibition of polyamine synthesis induced increased formation of $GlcNH_3^+$ residues, and g) addition of ascorbate to this rhGpc-1 PG resulted in NO-dependent auto-degradation of a major portion of the $GlcNH_3^+$ residues in the HS chains indicating endogenous S-nitrosylation of Gpc-1 PG. On

the basis of these results we propose that S-nitrosylation of rhGpc-1 core protein and formation of $GlcNH_3^+$ residues in the HS chain take place during biosynthesis and secretion. However, it cannot be excluded that endogenous Gpc-1 can become further S-nitrosylated during recycling. Inhibition of polyamine biosynthesis may modulate HS on Gpc-1 with increased amount of $GlcNH_3^+$ residues either by signaling to the biosynthetic machinery by activation of N-deacetylase/sulfotransferases (NDST) with high deacetylase activity or by affecting the degradative pathway by induction of sulfamidase resulting in increased potential polyamine binding sites and thereby stimulating polyamine uptake [14, 16]. The finding that disappearance of the 10E4 epitope in $CuCl_2$, SNP, and ascorbate treated PG was accompanied by similar size distribution supports this notion. The presence of $GlcNH_3^+$ in HS may further stimulate S-nitrosylation.

HEK 293 cells reportedly express no or little NO synthases and produce small amounts of NO [24, 25]. However, we could detect NO in Gpc-1 transfected EBNA 293 by adding DAF-2 to the cells and analyzing them with confocal fluorescene microscopy and FACS (see Supplemental data). Spatial regulation of NO synthases has been suggested as a way to regulate S-nitrosylation. NO synthases are not known to localize to the lumen of ER or Golgi. However, S-nitrosylated proteins residing in ER or Golgi have been found [6]. NO synthase present at the cytosolic face of the Golgi membrane may synthesize NO that diffuses through the membrane and S-nitrosylates proteins.

The $GlcNH_3^+$ residues found in native HS structures have been implicated in several important cell biological processes. HS chains containing $GlcNH_3^+$ units have been found in scrapie lesions of scrapie infected mice [26, 27]. Furthermore, $GlcNH_3^+$ units can be targeted for 3-O-sulfation generating cell surface HS functioning as an entry receptor for herpes simplex virus-1 [28, 29]. S-Nitrosylated recycling Gpc-1 with HS chains containing $GlcNH_3^+$ may be involved in different pathophysiological processes. Several neurological disorders illustrate the importance of Gpc-1 recycling, adequate HS turnover and deaminative cleavage of HS chains. Recent studies from this laboratory have shown that deaminative cleavage of HS is abrogated in Niemann-Pick C1 disease but can be restored by exogenously supplied ascorbate [13]. Other recent studies from our laboratory have demonstrated involvement of Gpc-1 and its NO-dependent autoprocessing in scrapie infection [30]. We have shown that scrapie infection induces de-nitrosylation of Gpc-1 thereby promoting deaminative cleavage of its HS chains at $GlcNH_3^+$ and liberation of HS oligosaccharides that form conjugates with misfolded scrapie protein [30]. Furthermore, we have shown that HS degradation products originating from NO-dependent autoprocessing of Gpc-1 associate with oxidized proteins in proteasomes [31]. Silencing of Gpc-1 expression or absence of NO-dependent autoprocessing resulted in accumulation of oxidized proteins, suggesting that the HS degradation products released from Gpc-1 are involved in the clearance of misfolded proteins [31]. The endogenous trigger that induces release of NO from S-nitrosylated Gpc-1 core protein remains however unknown.

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