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The Structural Role of N-Linked Glycans on Human Glypican-1

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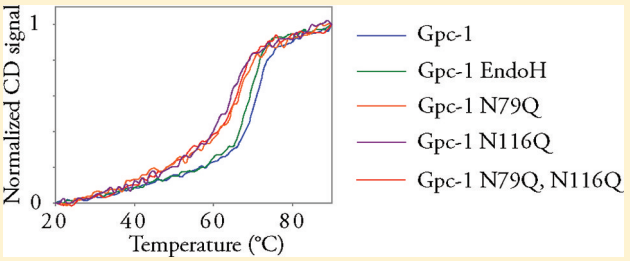
Supporting Information

ABSTRACT: Glypicans are cell-surface heparan sulfate proteoglycans that regulate developmental signaling pathways by binding growth factors to their heparan sulfate chains. The primary structures of glypican core proteins contain potential N-glycosylation sites, but the importance of N-glycosylation in glypicans has never been investigated in detail. Here, we studied the role of the possible N-glycosylation sites at Asn-79 and Asn-116 in recombinant anchorless glypican-1 expressed in eukaryotic cells. Mutagenesis and enzymatic cleavage indicated that the potential N-glycosylation sites are invariably occupied. Experiments using the drug tunicamycin to inhibit the N-linked glycosylation of glypican-1 showed that secretion of anchorless glypican-1 was reduced and that the protein did not accumulate inside the cells. Heparan sulfate substitution of N-glycosylation mutant N116Q was similar to wild-type glypican-1 while the N79Q mutant and also the double mutant N79Q,N116Q were mostly secreted as high-molecular-weight heparan sulfate proteoglycan. N-Glycosylation mutants and N-deglycosylated glypican-1 had far-UV circular dichroism and fluorescence emission spectra that were highly similar to those of N-glycosylated glypican-1. A single unfolding transition at high concentrations of urea was found for both N-deglycosylated glypican-1 and glypican-1 in which the N-glycosylation sites had been removed by mutagenesis when chemical denaturation was monitored by circular dichroism and fluorescence emission spectroscopy. In summary, we have found that the potential N-glycosylation sites in glypican-1 are invariably occupied and that the N-linked glycans on glypican-1 affect protein expression and heparan sulfate substitution but that correct folding can be obtained in the absence of N-linked glycans.



Glycosaminoglycans (GAGs) are long, unbranched polysaccharides that are usually covalently attached to a protein core, forming a proteoglycan (PG). Genetic studies in *Drosophila melanogaster* (the fruit fly) have revealed that loss of function in GAG synthesis mimics loss of function in developmentally important growth factor signaling pathways, suggesting a role for the GAGs in morphogen regulation.[1] Fruit flies defective in the enzyme responsible for the elongation of the GAG heparan sulfate (HS), or lacking the heparan sulfate proteoglycans (HSPGs) Dally and Dally-like protein, are defective in hedgehog growth factor signaling.[2,3] Dally and Dally-like protein belong to the glypican family of cell-surface HSPGs, six members of which have been found in mammals (Gpc-1 to Gpc-6). All glypicans share an N-terminal signal peptide that is responsible for translocation to the endoplasmatic reticulum (ER) and a C-terminal signal peptide that is responsible for glycosylphosphatidylinositol (GPI) anchorage. Glypicans are also characterized by having 14 evolutionarily conserved Cys residues and an HS attachment domain near the C-terminus. The specific spatial and temporal expression of glypicans during mammalian development suggests that each glypican serves a specific role during morphogenesis.[4] The two most well-studied mammalian glypicans are glypican-1 (Gpc-1) and glypican-3 (Gpc-3). Gpc-1 is expressed in neural tissue, and Gpc-1 knockout mice have reduced brain size at birth due to defective fibroblast growth factor signaling.[5] Gpc-3 is expressed in most tissues during development, except in the nervous system, and is mutated in patients with Simpson–Golabi–Behmel syndrome, an overgrowth disorder.[6,7] Although many of the functions of glypicans are related to their HS chains, it has recently been shown that hedgehog signaling in Dally-like protein-deficient cells can be rescued by expression of Dally-like protein without HS chains, suggesting a role for the core protein itself.[8] Moreover, other studies have shown that glypican core proteins can bind to hedgehog, bone morphogenetic protein 4, decapentaplegic,[9] and Wnt.[10] There is no detailed structural information regarding the interaction between glypican core proteins and growth factors.

We have previously shown that anchorless recombinant human Gpc-1 (rhGpc-1) is a stable protein that can resist high concentrations of the chemical denaturants urea and guanidine-HCl.[11] Unfolding occurred in a single step, and unfolding data were closely fitted by a two-state model. These results suggested that Gpc-1 core protein is a densely packed globular protein. Spectroscopic studies comparing Gpc-1 with and without HS chains revealed no difference in conformation,

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suggesting that the HS chains do not influence the folding of the core protein. Moreover, refolding of heat-denatured Gpc-1 without HS chains was found to be dependent on protein concentration, suggesting that intermolecular interactions are involved in irreversible denaturation. However, refolding was concentration independent for the HS-substituted form, suggesting that electrostatic repulsion between the acidic core protein and polyanionic HS chains prevented irreversible aggregation.

N-linked glycosylation is a common post-translational protein modification in eukaryotic cells.[12] In the ER, a core oligosaccharide can be added to an Asn residue in the sequon Asn-X-Thr/Ser, where X is any amino acid except Pro. The N-glycosylation sequon is necessary but not sufficient for glycosylation. Subsequent trimming and modification of the core oligosaccharide to form hybrid or complex N-linked glycans can occur in the ER and Golgi apparatus. Among other functions, N-linked oligosaccharides are involved in quality control of protein synthesis and in intracellular transport and targeting of proteins. The importance of N-linked oligosaccharides in the glypican family of HSPGs has never been investigated in detail. The attachment sites for N-glycosylation in glypicans are not evolutionarily conserved, like many of the Cys residues (Supporting Information Figure S1A,B), with the exception of Gpc-3 and Gpc-5, which share two potential attachment sites for N-glycosylation. One of the potential attachment sites in Gpc-3 and Gpc-5 is also present in Dally. Interestingly, human Gpc-2 and Gpc-6 lack potential attachment sites for N-glycosylation, suggesting that N-linked glycans are not absolutely required in the biosynthesis of glypicans.

In the present work, we investigated the importance of N-linked glycans on Gpc-1 by removing the N-glycans either by enzymatic treatment or by mutagenesis. Folding and unfolding of Gpc-1 with or without N-linked glycans were monitored by circular dichroism (CD) and fluorescence emission spectroscopy, and expression and HS substitution of Gpc-1 N-glycosylation mutants were monitored by SDS-PAGE. We conclude that that the potential N-glycosylation sites in glypican-1 are invariably occupied and that the N-linked glycans on glypican-1 affect protein expression and HS substitution but that correct folding can be obtained in the absence of N-linked glycans.

EXPERIMENTAL PROCEDURES

Materials. Human Gpc-1 cDNA clone IMAGE ID 6275649 was obtained from Geneservice (UK). The expression vector pCEP-BM40-HisTEV and 293 human embryonic kidney (HEK293) cells were obtained as described previously.[11]

PfuII, DpnI, and all other restriction enzymes used were from Fermentas (Canada). PNGase F was purchased from Roche (Germany), maltose binding protein fusion-tagged endoglycosidase H was obtained from New England Biolabs (MA), and HS lyase (heparitinase) was from Seikagaku (Japan). Ni-NTA columns were from GE Healthcare Life Sciences (UK). Kifunensine, protein-free medium for CHO cells, and Coomassie blue were from Sigma-Aldrich (MO). Lipofectamine, 4−12% Bis-Tris SDS-PAGE gels, Ni-NTA agarose, and hygromycin B were from Invitrogen (CA).

Plasmid Construction and Mutagenesis. A library with mutated Gpc-1 cDNA was created using PCR-based site-directed mutagenesis. Gpc-1 cDNA (GenBank accession number BC051279) was amplified with degenerate primers using PfuII. The parental cDNA was digested with DpnI, and bacteria were transformed. Combinations of the mutations were created either by using mutated cDNA as PCR template or by digesting differently mutated cDNAs with appropriate restriction enzymes. Mutated Gpc-1 cDNA was amplified by PCR and introduced into the expression vector pCEP4-BM40-HisTEV as described previously.[11] The following degenerate primers were used: S486A,S488A,S490A, 5′-GACGACGGC**G**-**CA**GGC**G**CGGG**TGCA**GGTGATGGCTG-3′; N79Q, 5′-TGGAGGAGAACCTGGCC**CAG**CGCAGCCATGC-3′; N116Q, 5′-CAGCACCTGCTG**CAG**GACTCGGAGCG-GACG-3′. Mutated nucleotides are marked in boldface. The C to T mutation in primer S486A,S488A,S490A was done in order to relieve the primer from secondary structure and did not result in any change of amino acid. All mutations and constructs were verified by sequencing at Eurofins MWG Operon (Germany).

Transfection and Cloning of Cells. Transfection was performed using Invitrogen's standard protocol for transfection with Lipofectamine 2000. The cells were split 1:10 1 day after the transfection and hygromycin B was added to a concentration of 200 μg/mL on the following day. The cells were cloned by limiting dilution after several weeks of growth in the selective medium. Clones expressing a large amount of rhGpc-1 were expanded and used in this study.

Purification of rhGpc-1. Cells expressing rhGpc-1 were grown to confluence in minimal essential medium with Earle's salts supplemented with 10% (v/v) fetal bovine serum, 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 protein-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 50 mM sodium phosphate (pH 8.0) containing 0.3 M NaCl. The dialyzed conditioned medium was applied to a Ni-NTA column, which was washed with 0.3 M NaCl, 50 mM sodium phosphate, and 10 mM imidazole (pH 8.0), and the recombinant Gpc-1 was eluted using a linear imidazole gradient (0−250 mM imidazole). Alternatively, conditioned medium was dialyzed against 0.3 M NaCl, 50 mM sodium phosphate, and 10 mM imidazole (pH 8.0) and then incubated with Ni-NTA agarose on a rocker at 8 °C for 4 h. Recombinant Gpc-1 was purified by washing the Ni-NTA agarose with 0.3 M NaCl, 50 mM sodium phosphate, and 10 mM imidazole (pH 8.0) and eluted using the same buffer containing 250 mM imidazole.

Tunicamycin and Kifunensine Treatment of Gpc-1 Producing Cells. HEK293 cells expressing His_6-Gpc-1 ΔHS were grown to confluence in the wells of a 24-well plate. The cells were washed three times with PBS, and protein-free medium with varying concentrations of tunicamycin (0−2.0 μg/mL) was added to the wells. After 3 h, the medium was replaced with medium with the same concentration of tunicamycin, and the cells were incubated for another 24 or 48 h, after which the cell medium was harvested. Kifunensine was dissolved in water to a concentration of 5 mM. This stock solution was stored frozen and diluted to 10 μM in protein-free medium for treatment of Gpc-1 producing cells.

For toxicity assay, HEK293 cells expressing His6-Gpc-1 ΔHS were seeded into 96-well microculture plates at 50 000 cells/well in MEM supplemented with 10% fetal bovine serum. After 24 h of plating, the cells were cultured in protein-free medium containing 1.5 μg/mL tunicamycin or 10 μM kifunensine for 24 h (n = 6). Controls without drug treatment were included.

Cells were then fixed in 1% glutaraldehyde dissolved in Hanks' balanced salt solution for 15 min, and the nuclei were then stained with 0.1% crystal violet (aqueous). After washing and cell lysis for 24 h in Triton X-100, the amount of bound dye was quantified by measuring the absorbance at 600 nm in a microplate photometer (Titertek multiscan).

Western Blot Analysis. Cells expressing His_6-Gpc-1 ΔHS were incubated either without or with 1.5 μg/mL tunicamycin for 24 h, after which the conditioned medium was harvested and the cells were lysed in radioimmunoprecipitation assay buffer: 0.1% (w/v) sodium dodecyl sulfate, 0.5% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate in PBS supplemented with 0.5 mM phenylmethyl sulfonyl fluoride at 4 °C. The cell lysates were incubated on a rocker for 30 min, after which they were centrifuged at 15000*g* for 20 min, also at 4 °C. The protein concentration of the supernatant was determined using the bicinchoninic acid protein assay. Conditioned media and cell extracts were analyzed by adding 17.5 μL of conditioned medium or 20 μg of cell extract to each well in a 4−12% Bis-Tris SDS-PAGE gel. After electrophoresis, the gel was blotted onto a poly(vinylidene fluoride) membrane. The membrane was blocked by gentle shaking of the membrane with 5% (w/v) nonfat dry milk in PBS with 0.05% (v/v) Tween-20 for 1 h at room temperature. The primary antibody was polyclonal antihuman Gpc-1; this was diluted 1:5000 in PBS with 0.05% (v/v) Tween-20 and incubated overnight at 4 °C with gentle shaking of the membrane. The membrane was then washed extensively with PBS with 0.05% (v/v) Tween-20, and secondary antibody was added. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (Promega); this was diluted to 0.1 μg/mL in PBS with 0.05% (v/v) Tween-20 and incubated for 45 min at room temperature. After extensive washing in PBS with 0.05% (v/v) Tween-20, the blot was developed using a LAS-1000 imaging system (Fujifilm).

Enzymatic Treatments. HS lyase digestion was performed in 10 mM HEPES, 3 mM $Ca(OAc)_2$, and 0.1% (v/v) Triton X-100 (pH 7.0) using 0.5 milliunits of enzyme per 20 μg of His6-Gpc-1 at 37 °C for 2 h. Digestion with PNGase F and endoglycosidase H was performed by adding SDS and dithiothreitol at final concentrations of 0.5% and 40 mM, respectively, to the HS lyase-digested recombinant Gpc-1 and then boiling the samples for 10 min. The samples were cooled and divided into two, and sodium citrate was added to a final concentration of 50 mM (pH 5.5) for samples that were digested with 500 units endoglycosidase H. NP-40 and Tris-HCl (pH 8.0) were added to final concentrations of 1% (v/v) and 50 mM, respectively, for the samples that were digested with 0.5 U of PNGase F. The samples were incubated at 37 °C for 3 h. Endoglycosidase H treatment of 20 μg of native rhGpc-1 that had been produced in the presence of kifunensine was performed in 25 mM sodium phosphate (pH 7.0) at 37 °C for 3 h with 1000 units of endoglycosidase H. For the purpose of conformational studies, a large amount of rhGpc-1 produced from kifunensine-treated cells was treated with endoglycosidase H overnight at 37 °C. Endoglycosidase H was removed from the samples by repeating the Ni-NTA purification of Gpc-1. A similar approach was used for HS lyase treatment of His_6-Gpc-1 N79Q and Gpc-1 N116Q prior to heat denaturation studies. Triton X-100 was omitted from the HS lyase cleavage buffer, digestion was performed overnight at 37 °C, and the enzyme was removed by repeating the Ni-NTA purification.

CD Spectroscopy and Heat Denaturation. Far-UV CD spectra were recorded using a Jasco J-810 spectropolarimeter equipped with a Peltier thermostated cell holder. A 2 mm quartz cuvette was used, and the measurement range was 190−250 nm. The cuvette was thermostated at 20 °C unless otherwise stated. The following parameters were used: sensitivity, 100 mdeg; data pitch, 1 nm; scan rate, 100 nm/min; response time, 4 s; bandwidth, 1 nm; and accumulation, 2. Baseline spectra were recorded for pure buffer and subtracted from the corresponding protein spectra. Stock solutions of rhGpc-1 were clarified by centrifugation at 15000*g* for 15 min, and protein concentration was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). Bovine serum albumin was used as a protein standard. Samples were prepared by diluting the rhGpc-1 stock solution to 0.1 mg/mL in 20 mM sodium phosphate buffer (pH 7.4). Mean residue ellipticity was calculated using 110.5 as the mean residue molecular weight of Gpc-1. The following parameters were used to study heat denaturation of rhGpc-1 monitored by CD: wavelength, 208 nm; sensitivity, 100 mdeg; data pitch, 1 °C; delay time, 1 min; response, 4 s; bandwidth, 1 nm. Heat denaturation studies were also performed in a 2 mm quartz cuvette. Samples of rhGpc-1 at 0.1 mg/mL were heated from 20 to 90 °C at 1 °C/min, and CD spectra were measured at 20 °C, at 90 °C, and again at 20 °C after the samples had been cooled.

Fluorescence Spectroscopy. Fluorescence spectra were measured using a Jasco J-810 spectropolarimeter. A 2 × 10 mm quartz cuvette was used. The excitation wavelength was 280 nm, and emission was recorded between 310 and 400 nm. The sensitivity was 600 V; data pitch, 1 nm; response time, 0.5 s; bandwidth, 5 nm; and accumulation, 2. The cuvette was thermostated at 20 °C. The protein concentration was 0.1 mg/mL in 20 mM sodium phosphate (pH 7.4).

Chemical Denaturation. Urea denaturation curves were obtained by monitoring the CD and fluorescence of 0.1 mg/mL rhGpc-1 in buffer with different concentrations of urea. Solutions with 30 different concentrations of urea ranging from 0 to 9 M were prepared by diluting a stock solution of 10 M urea, 20 mM sodium phosphate (pH 7.0) with 20 mM sodium phosphate (pH 7.0). The pH of the different preparations was adjusted, and the protein was diluted to 0.1 mg/mL from a stock solution. These samples were allowed to equilibrate overnight at room temperature, after which the CD signal was measured at 222 nm for 1 min (the data obtained were averaged) and the fluorescence intensity was measured at 320 nm with an excitation wavelength of 280 nm. The sensitivity was 600−650 V for the fluorescence measurements. The sensitivity was changed between different denaturation curves to compensate for small differences in protein concentration. CD was measured in a 2 mm path length quartz cuvette, and fluorescence was measured in a 2 × 10 mm cuvette.

Analysis of Denaturation Data. The data from the urea denaturation curves were analyzed as described previously using a linear extrapolation method assuming reversible two-state unfolding.[11] In brief, the following equation was fit to the data:

$$Y_0 = \frac{(k_N[D] + b_N) + (k_U[D] + b_U)e^{-(\Delta G_{NU}(H_2O) - m_D[D])/RT}}{1 + e^{-(\Delta G_{NU}(H_2O) - m_D[D])/RT}} \quad (1)$$

where the slopes and *y*-intercepts of the pre- and post-transition baselines are represented by k_N, k_U, b_N, and b_U, respectively. [D] is the denaturant concentration, R is the molar gas constant, and T is the temperature. $\Delta G_{NU}(H_2O)$ is the free

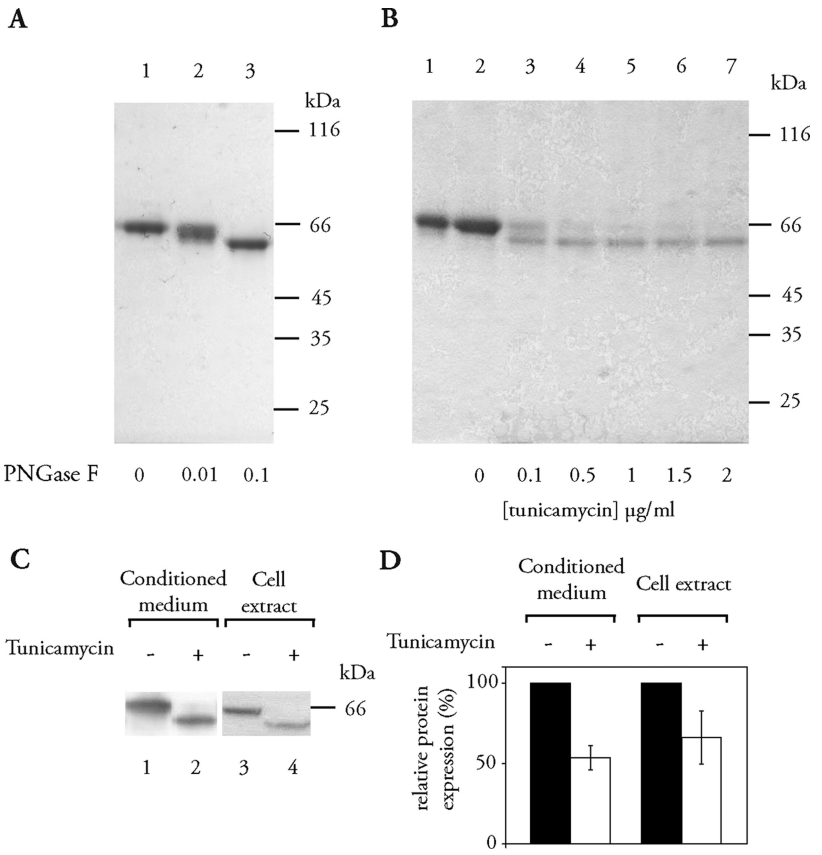

Figure 1. PNGase F digestion of His_6-Gpc-1 ΔHS and inhibition of expression by tunicamycin treatment. (A) Ni-NTA purified His_6-Gpc-1 ΔHS was incubated for 1 h at 37 °C with increasing concentrations of PNGase F and analyzed on a Coomassie blue-stained SDS-PAGE gel. 1 μg of purified protein was added to each well. Lane 1, untreated. Lane 2, 0.01 U PNGase F. Lane 3, 0.1 U PNGase F. (B) Cells expressing His_6-Gpc-1 ΔHS were incubated in the presence or absence of tunicamycin for 48 h as described in Experimental Procedures. Conditioned medium was analyzed using SDS-PAGE, and the gel was stained with Coomassie blue. Ni-NTA-purified His_6-Gpc-1 ΔHS was included as a control in lane 1. (C) Cells expressing His_6-Gpc-1 ΔHS were incubated without or with 1.5 μg/mL tunicamycin, and conditioned media and cell extracts were probed using anti-hGpc-1 in a Western blot analysis. Representative Western blot images are shown (n = 4). (D) The amount of protein in the Western blot analysis was quantified using Gel-Pro Analyzer software. The data are presented as protein levels relative to the amounts in untreated conditioned media and cell extract.

energy change in the absence of denaturant, and m_D is the linear dependence of ΔG_{NU} on denaturant concentration. The software Kaleidagraph (Synergy Software) was used to fit the data using nonlinear least-squares analysis. The fitted data were normalized to the apparent fraction unfolded, F_{app}, using the equation

$$F_{app} = (Y_0 - Y_N)/(Y_U - Y_N) \quad (2)$$

For the heat denaturation experiments, the Gibbs−Helmholtz equiation was fitted to the data, as described.[13] The presented data were normalized by treating the lowest value in each data set as 0 and the largest as 1.

RESULTS

N-Glycosylation of Gpc-1. Gpc-1 contains two potential N-glycosylation sites at Asn-79 and Asn-116 and three attachment sites for O-glycosylation at Ser-486, Ser-488, and Ser-490 (Supporting Information Figure S2). To investigate N-glycosylation of Gpc-1, we expressed and purified histidine-tagged Gpc-1 lacking the GPI anchor and the HS attachment sites (His_6-Gpc-1 ΔHS) as previously described.[11] After purification, samples were treated under denaturing conditions with increasing concentrations of PNGase F, which removes Asn-bound N-linked glycans from glycoproteins almost regardless of the type of N-linked glycan present on the protein. The samples were analyzed on SDS-PAGE gel stained with Coomassie blue. For the untreated sample, a band of $M_r \sim$ 65 kDa was detected, corresponding in size to rhGpc-1 without HS chains but with N-linked glycans (Figure 1A, lane 1). Treatment of His_6-Gpc-1 ΔHS with low concentration of PNGase F yielded a band that appeared as a smear, indicating that N-linked glycans had been removed from His_6-Gpc-1 ΔHS but that the reaction had not gone to completion (Figure 1A, lane 2). At higher concentrations of PNGase F, a distinct band of $M_r \sim$ 60 kDa was seen (Figure 1A, lane 3), indicating that the reaction was complete and that all N-linked glycans had been removed. The appearance of a smear after incubation with small amounts of PNGase F (Figure 1A, lane 2) and the reduction in size of ~5 kDa when the reaction had gone to completion (Figure 1A, lane 3) indicated that the two potential N-glycosylation sites in Gpc-1 are occupied.

Effect of Inhibition of N-Glycosylation on the Biosynthesis of Gpc-1. In order to investigate the importance of N-glycosylation in the biosynthesis of Gpc-1, we treated cells expressing anchorless His_6-Gpc-1 ΔHS with the drug tunicamycin, which inhibits the enzyme UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-1-phosphate transferase and thus prevents N-glycosylation.[14] Confluent

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cells expressing His_6-Gpc-1 ΔHS were incubated in protein-free medium in the absence or presence of different concentrations of tunicamycin for 48 h. The culture medium was harvested and analyzed on SDS-PAGE gels stained with Coomassie blue (Figure 1B). Untreated cells produced a band of $M_r \sim 65$ kDa, corresponding in size to His_6-Gpc-1 ΔHS with N-linked glycans. At tunicamycin concentrations of 0.1 and 0.5 μg/mL, two bands appeared on the SDS-PAGE gel, one at $M_r \sim 65$ kDa and one at $M_r \sim 60$ kDa, the latter corresponding in size to PNGase F-treated His_6-Gpc-1 ΔHS, indicating that N-glycosylation was inhibited but that inhibition was not complete. At tunicamycin concentrations of 1 μg/mL or higher, the 60 kDa band was predominant, indicating almost complete inhibition of N-glycosylation on His_6-Gpc-1 ΔHS. Despite the reduced amount of protein produced by the cells following tunicamycin treatment, the experiment indicated that it is possible to express Gpc-1 without N-linked glycans. In order to investigate the effect of tunicamycin on cell survival, a cytotoxicity assay was performed after 24 h treatment with 1.5 μg/mL tunicamycin and showed 99% cell survival (data not shown). To be able to quantify how tunicamycin treatment affects the level of Gpc-1 production, His_6-Gpc-1 ΔHS producing cells were incubated in the absence or presence of 1.5 μg/mL tunicamycin in protein-free medium for 24 h. The conditioned medium was then harvested, and the cells were extracted using radioimmunoprecipitation assay buffer as described in Experimental Procedures. The cell extracts of tunicamycin-treated and untreated cells contained equal amounts of total protein, supporting that the 24 h tunicamycin treatment was not detrimental to the cells. The conditioned medium and the cell extracts were analyzed using Western blot with an antibody against human Gpc-1 (Figure 1C). As seen in Figure 1C, the 60 kDa band was predominant in the conditioned medium and cell extracts treated with 1.5 μg/mL tunicamycin (Figure 1C, lanes 2 and 4). The intensities of the bands were quantified using the software Gel-Pro Analyzer (Media Cybernetics) (Figure 1D). By this analysis, a ∼50% reduction in Gpc-1 expression was detected in both conditioned media and cell extracts upon incubation with tunicamycin, indicating that the folding of His_6-Gpc-1 ΔHS was slower in the absence of N-linked glycans since no accumulation of the protein inside the cells was detected.

Effect of Mutations of N-Glycosylation Sites in Gpc-1 on Secretion and HS Attachment. To further investigate whether the potential N-glycosylation sites are occupied and to be able to evaluate the effect of the individual N-linked glycan on the biosynthesis of Gpc-1, we disrupted the potential N-glycosylation sites using site-directed mutagenesis by replacing the N-glycosylation Asn residues at positions 79 and 116 with Gln in different combinations in rhGpc-1 with intact HS attachment sites (His_6-Gpc-1 N79Q, His_6-Gpc-1 N116Q, and His_6-Gpc-1 N79Q,N116Q; see also Table 1 for a complete list of rhGpc-1 glycosylation mutants used in the study). Cells were transfected with the mutant constructs, grown in selective medium for several weeks, and then cloned by limiting dilution. The conditioned medium from the clones was analyzed by SDS-PAGE and high-producing clones were further expanded and used in the study (data not shown). The same numbers of cells ($\sim 10^6$) were grown to confluence in parallel, washed with PBS, and then maintained in protein-free medium for 72 h. The different rhGpc-1 N-glycosylation mutants and also wild-type His_6-Gpc-1 with all glycosylation sites intact were purified from 50 mL conditioned medium using Ni-NTA affinity chromatography as described in Experimental Procedures, and the quantities of the expressed proteins were measured by BCA protein assay. The amount of purified protein from His_6-Gpc-1 N79Q,N116Q transfected cells was approximately one-tenth of the amount purified from cells expressing wild-type protein, and His_6-Gpc-1 N79Q transfected cells produced half the amount compared to wild-type transfected cells (data not shown). His_6-Gpc-1 N116Q transfected cells produced similar amounts of protein to that produced by wild-type His_6-Gpc-1. The purified proteins were then visualized in an SDS-PAGE gel stained with Coomassie blue before (Figure 2A)

Table 1. Glycosylation Mutations in Recombinant Gpc-1

mutations of amino acids	recombinant anchorless Gpc-1 as referred to in text
–	wild-type His_6-Gpc-1
N79Q	His_6-Gpc-1 N79Q
N116Q	His_6-Gpc-1 N116Q
N79Q,N116Q	His_6-Gpc-1 N79Q,N116Q
S486A,S488A,S490A	His_6-Gpc-1 ΔHS
N79Q,N116Q,S486A,S488A,S490A	His_6-Gpc-1 N79Q,N116Q ΔHS

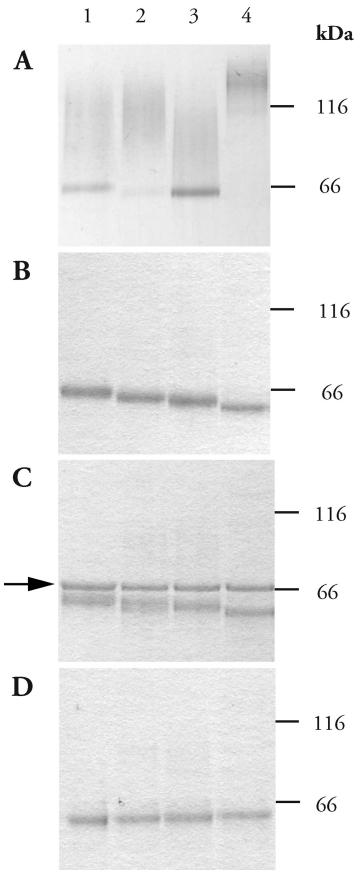

Figure 2. SDS-PAGE analysis of recombinant Gpc-1 with deleted potential N-glycosylation sites. Anchorless His_6-Gpc-1 was Ni-NTA purified from conditioned medium from stable cell lines expressing wild-type or N-glycosylation mutant Gpc-1 and analyzed on a Coomassie blue-stained SDS-PAGE gel (panel A). Wild-type His_6-Gpc-1 (lane 1), His_6-Gpc-1 N79Q (lane 2), His_6-Gpc-1 N116Q (lane 3), and His_6-Gpc-1 N79Q,N116Q (lane 4). The purified proteins were digested with HS lyase (panel B), HS lyase and endoglycosidase H (panel C, endoglycosidase H is marked with an arrow), or HS lyase and PNGase F (panel D) and analyzed on a Coomassie blue-stained SDS-PAGE gels. 1 μg of purified protein was added to each well.

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and after treatment with HS lyase (Figure 2B), or after treatment with HS lyase and endoglycosidase H (Figure 2C), or after treatment with HS lyase and PNGase F (Figure 2D). As previously shown,[11] wild-type His_6-Gpc-1 was expressed both as PG (M_r ~ 65−120 kDa) and core protein (M_r ~ 65 kDa) (Figure 2A, lane 1). Surprisingly, His_6-Gpc-1 N79Q was mostly expressed as a PG smear (M_r ~ 65−120 kDa), and for His_6-Gpc-1 N79Q,N116Q, only a high-molecular-weight PG smear of M_r ~ 120 kDa was detected (Figure 2A, lanes 2 and 4). His_6-Gpc-1 N116Q migrated similarly to the wild-type His_6-Gpc-1, but a slight reduction in the size of the core protein band was seen (Figure 2A, lane 3). When these mutant proteins were treated with HS lyase, the PG smears disappeared and the core protein bands of ~60−65 kDa were detected, indicating degradation of HS chains on all proteins (Figure 2B). Compared to wild-type His_6-Gpc-1 core protein, a small reduction in size was detected for His_6-Gpc-1 N79Q and His_6-Gpc-1 N116Q, consistent with the removal of one N-glycosylation site (Figure 2B; compare lanes 2 and 3 with lane 1). A further reduction in the size of the His_6-Gpc-1 N79Q,N116Q band was consistent with the removal of both N-glycosylation sites (Figure 2B, lane 4). Endoglycosidase H removes high mannose or hybrid—but not complex—N-linked glycans from glycoproteins, and the cleavage occurs between the two *N*-acetylglucosamine residues in the N-linked glycan core, leaving one *N*-acetylglucosamine residue on the treated protein. Treatment of wild-type His_6-Gpc-1 with both HS lyase and endoglycosidase H only yielded a slightly broader band compared to that of the HS lyase digested material, indicating that most of the N-linked glycans on wild-type Gpc-1 are of complex type and therefore insensitive to endoglycosidase H (Figure 2C, lane 1). The upper band in Figure 2C represents the endoglycosidase H enzyme with M_r of 70 kDa. Digestion of His_6-Gpc-1 N79Q and His_6-Gpc-1 N116Q with HS lyase and endoglycosidase H produced slightly broader protein bands (Figure 2C, lanes 2 and 3). To further confirm that the N-linked glycans on Gpc-1 are of complex type, native His_6-Gpc-1 ΔHS was treated with the enzyme sialidase, which removes terminal sialic acid from complex N-linked glycans. His_6-Gpc-1 ΔHS treated with sialidase showed a reduced number of bands on a native PAGE gel stained with Coomassie blue as compared to untreated samples, indicating that sialic acid on the complex N-linked glycan contributes to the charge heterogeneity of Gpc-1 (data not shown). As shown in Figure 2D, when the mutant proteins were treated with both HS lyase and PNGase F, all lanes showed protein bands of the same size (M_r ~ 60 kDa), indicating complete removal of glycans from rhGpc-1. No further reduction in size was seen for the double mutant His_6-Gpc-1 N79Q,N116Q, indicating that removal of the N-glycosylation sites in Gpc-1 had completely abolished N-glycosylation.

Expression of Nonglycosylated and Deglycosylated Native Gpc-1. To study the effect of lack of glycosylation on the folding and conformation of Gpc-1, we expressed rhGpc-1 lacking both N- and O-glycosylation sites (His_6-Gpc-1 N79Q,N116Q ΔHS) and produced deglycosylated His_6-Gpc-1 ΔHS, in which the N-linked glycans had been removed enzymatically under native conditions. We first tried to remove the N-linked glycans on His_6-Gpc-1 ΔHS enzymatically using PNGase F under native conditions. However, 72 h of incubation at 37 °C in the presence of mild detergent and large amounts of PNGase F were needed to remove the N-linked glycans from Gpc-1 in this situation. Since a large quantity of protein was needed for conformational studies, it was not feasible to use this method. In another attempt to produce deglycosylated Gpc-1, we treated the His_6-Gpc-1 ΔHS producing cells with the plant alkaloid kifunensine, which inhibits Golgi α-mannosidases, making all N-linked glycans of high-mannose type and sensitive to endoglycosidase H.[15] First a cytotoxicity assay after 24 h treatment with 10 μM kifunensine was performed and indicated complete cell survival upon kifunensine treatment under these conditions. Then collected His_6-Gpc-1 ΔHS purified from conditioned medium from cells treated with 10 μM kifunensine was treated with endoglycosidase H under native conditions and analyzed on SDS-PAGE gels stained with Coomassie blue. A band of M_r ~ 60 kDa appeared for the endoglycosidase H treated His_6-Gpc-1 ΔHS (Figure 3, lane 1), indicating that kifunensine treatment

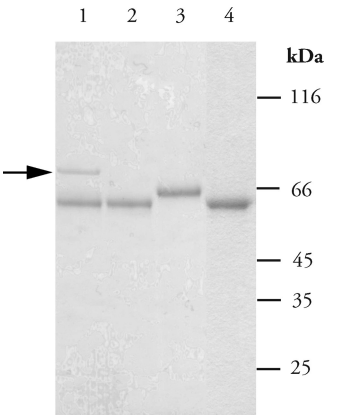

Figure 3. Endoglycosidase H treatment of recombinant Gpc-1 produced in the presence of kifunensine. His_6-Gpc-1 ΔHS-transfected cells were incubated in protein-free culture medium with 10 μM kifunensine, and His_6-Gpc-1 ΔHS was Ni-NTA purified from the conditioned medium. The protein was digested with endoglycosidase H and analyzed on Coomassie blue-stained SDS-PAGE gel (lane 1, endoglycosidase H is marked with an arrow). His_6-Gpc-1 ΔHS digested with PNGase F under denaturing conditions was used as a control (lane 2) together with untreated His_6-Gpc-1 ΔHS (lane 3). Also shown is Ni-NTA-purified His_6-Gpc-1 N79Q,N116Q ΔHS (lane 4). 1 μg of purified protein was added to each well.

makes the N-linked glycans on Gpc-1 sensitive to endoglycosidase H. The upper band in Figure 3, lane 1, represents endoglycosidase H. His_6-Gpc-1 ΔHS treated with PNGase F under denaturing conditions and untreated Gpc-1 ΔHS were used as controls (Figure 3, lanes 2 and 3, respectively). The mutant His_6-Gpc-1 N79Q,N116Q ΔHS, which lacked both N- and O-glycosylation sites, produced a band similar to His_6-Gpc-1 ΔHS treated with endoglycosidase H under native conditions and His_6-Gpc-1 ΔHS treated with PNGase F under denaturing conditions (Figure 3, lane 4), indicating that it is possible to express Gpc-1 without any glycans present during synthesis. This is consistent with the tunicamycin treatment shown in Figure 1.

Folding of Gpc-1 without N-Linked Glycans. To determine whether Gpc-1 lacking one or more N-glycosylation sites is folded correctly, we recorded CD spectra of the mutant glycoforms of rhGpc-1 in 20 mM sodium phosphate (pH 7.4) at 20 °C. N-deglycosylated His_6-Gpc-1 ΔHS, in which the N-linked glycans had been removed by kifunensine and endoglycosidase H treatment, was also investigated. The CD spectrum of His_6-Gpc-1 ΔHS exhibited a peak around 195 nm

and two troughs at 208 and 222 nm (Figure 4A, circles), as reported previously.[11] The previous results indicated that the presence of HS chains on Gpc-1 does not affect the CD spectrum of Gpc-1 but that the HS chains protect the core protein from heat-induced irreversible aggregation.[11] Here, we found that the CD spectrum of N-deglycosylated His_6-Gpc-1 ΔHS that had been treated with endoglycosidase H (Figure 4B, circles) and the CD spectrum of nonglycosylated His_6-Gpc-1 N79Q,N116Q ΔHS (Figure 4C, circles) were identical to the spectrum of His_6-Gpc-1 ΔHS (compare Figure 4B,C with Figure 4A, circles). Also, the spectra of the single mutants His_6-Gpc-1 N79Q and His_6-Gpc-1 N116Q were identical to the spectrum of His_6-Gpc-1 ΔHS (data not shown). The spectral identity between the different forms of rhGpc-1 implies that the presence of N-linked glycans does not affect the secondary structure.

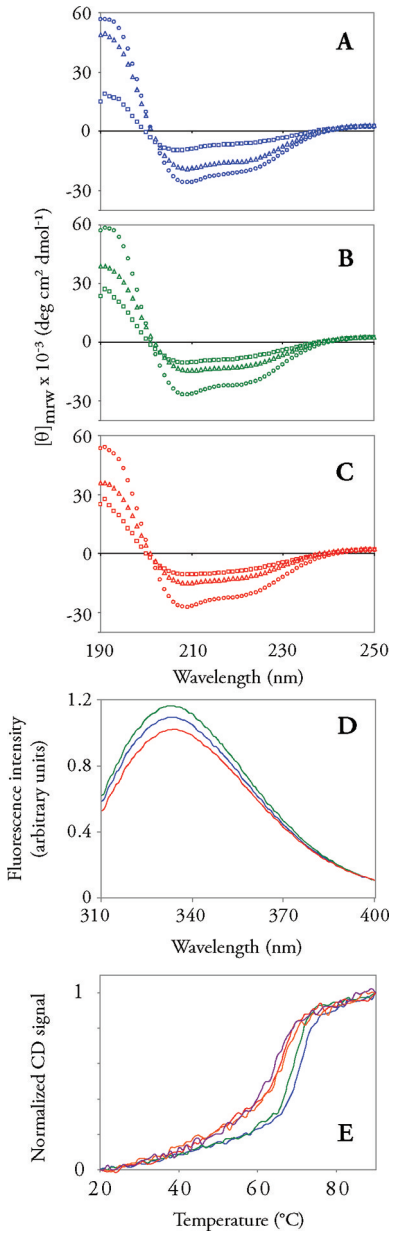

Figure 4. Conformation and stability of recombinant Gpc-1 with and without N-linked glycans monitored by CD and fluorescence emission spectroscopy. (A–C) Far-UV CD spectra of His_6-Gpc-1 ΔHS (A), endoglycosidase H-treated His_6-Gpc-1 ΔHS (B), and His_6-Gpc-1 N79Q,N116Q ΔHS (C) in 20 mM sodium phosphate (pH 7.4). The spectra were measured at 20 °C (circles), at 90 °C (squares), or after cooling from 90 to 20 °C (triangles). (D) Fluorescence emission spectra of His_6-Gpc-1 ΔHS (blue line), endoglycosidase H-treated His_6-Gpc-1 ΔHS (green line), and His_6-Gpc-1 N79Q,N116Q ΔHS (red line) in 20 mM sodium phosphate (pH 7.4). The excitation wavelength was 280 nm. All spectra were measured at 20 °C. (E) Heat denaturation of His_6-Gpc-1 ΔHS (blue line), endoglycosidase H-treated His_6-Gpc-1 ΔHS (green line), His_6-Gpc-1 N79Q,N116Q ΔHS (red line), His_6-Gpc-1 N79Q (orange), and His_6-Gpc-1 N116Q (purple, the HS chains were enzymatically removed from the two last protein species before the experiment) in 20 mM sodium phosphate (pH 7.4) monitored by ellipticity at 208 nm. The heating rate was 1 °C/min, and the data presented are the mean values of two independent heat-denaturation experiments using different batches of protein. The data were normalized to compensate for small differences in protein concentration.

Gpc-1 contains six Trp and 11 Tyr residues, which make it possible to study the tertiary structure by fluorescence emission spectroscopy. Fluorescence emission spectra of the same rhGpc-1 forms as shown in Figure 4A–C were recorded at 20 °C in 20 mM sodium phosphate, pH 7.4 (Figure 4D). The excitation wavelength was set to 280 nm to excite both Trp and Tyr. The fluorescence emission maximum was close to 333 nm for all investigated forms of rhGpc-1: His_6-Gpc-1 ΔHS (Figure 4D, blue line), N-deglycosylated His_6-Gpc-1 ΔHS (Figure 4D, green line), and nonglycosylated His_6-Gpc-1 N79Q,N116Q ΔHS (red line), indicating highly similar tertiary structure.

Thermal and Chemical Unfolding of Gpc-1 with and without N-Linked Glycans. We have previously shown that single structural transition occurs for rhGpc-1 around 70 °C.[11] By differential scanning calorimetry analysis, this transition does not follow a two-state mechanism for His_6-Gpc-1 ΔHS. To further explore this, we heated His_6-Gpc-1 ΔHS as well as the His_6-Gpc-1 N79Q and His_6-Gpc-1 N116Q mutants from 20 to 90 °C and recorded CD spectra at every 5 or 10 °C (Figure S3A–C). Since the HS chains on Gpc-1 affect the apparent T_m of heat denatured rhGpc-1, we removed the HS chains from the proteins using HS lyase prior to the heat denaturation experiments. For His_6-Gpc-1 N79Q and His_6-Gpc-1 N116Q without HS, the CD spectra reveal an isodichroic point at 202 nm, indicating that heat denaturation follows a two-state unfolding, whereas for His_6-Gpc-1 ΔHS the overlap at 202 nm is not perfect, suggesting the existence of some intermediate. To further investigate if heat denaturation of His_6-Gpc-1 ΔHS is reversible, we heated His_6-Gpc-1 ΔHS to 80 °C and recorded CD spectra before heating at 20 °C, at 80 °C, and when cooled down to 20 °C (Figure S4). Most of the signal was regained (more than 90%) upon cooling of the sample, and the CD spectrum at 20 °C after cooling the sample from 80 °C has very similar same shape as the initial spectrum recorded at 20 °C. In summary, heat denaturation His_6-Gpc-1 ΔHS does not follow a perfectly reversible two-state mechanism. Although a complete thermodynamic analysis of the heat unfolding cannot be performed, heat denaturation can still be used to evaluate the apparent thermal stability of the Gpc-1 N-linked glycan mutants. In order to this, we heated anchorless Gpc-1 with and without N-linked glycans to 90 °C at a rate of 1 °C/min and monitored the CD signal at 208 nm, where the largest difference in signal between native and denatured Gpc-1 occurred.[11] For His_6-Gpc-1 ΔHS, we observed a sharp decrease in signal around 70 °C, with a fitted T_m of 71 °C, indicating that Gpc-1 is 50% unfolded at this temperature (Figure 4E, blue line). His_6-Gpc-1 ΔHS with N-linked glycans removed by endoglycosidase H also displayed a sharp decrease in signal around 70 °C (Figure 4E, green line) with a fitted T_m of 69 °C.

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Nonglycosylated His_6-Gpc-1 N79Q,N116Q ΔHS showed a transition around 65 °C (Figure 4E, red line) with a fitted T_m of 66 °C, indicating that Gpc-1 synthesized without any glycans is slightly destabilized as compared to His_6-Gpc-1 ΔHS and to endoglycosidase H N-deglycosylated His_6-Gpc-1 ΔHS. Loss of CD signal for His_6-Gpc-1 N79Q,N116Q ΔHS was seen already at 50 °C, and there was a shoulder in the heat denaturation curve, indicating the presence of folding intermediates. To investigate whether this was an effect of a unique N-glycan, we performed identical experiments for His_6-Gpc-1 N79Q and His_6-Gpc-1 N116Q. The single mutants exhibited the same heat denaturation profiles as for His_6-Gpc-1 N79Q,N116Q ΔHS with fitted T_m of 66 and 64 °C for His_6-Gpc-1 N79Q and His_6-Gpc-1 N116Q, respectively, indicating that removal of a single N-glycan is enough to destabilize Gpc-1 but that removal of two gives no further destabilization. In order to study if the N-linked glycans affect the refolding of Gpc-1, the CD spectra of His_6-Gpc-1 ΔHS, endoglycosidase H treated His_6-Gpc-1 ΔHS and His_6-Gpc-1 N79Q,N116Q ΔHS were recorded at 20 °C (Figure 4A–C, circles), at 90 °C (squares), and after cooling the sample to 20 °C (triangles). Most of the CD signal was lost at 90 °C, but the CD spectra do not display a typical random coil appearance with a trough around 200 nm, indicating that some residual structure may be present even at this high temperature. Upon cooling of His_6-Gpc-1 ΔHS, 57% of the signal was regained for the trough at 208 nm, but only 23% and 25% of the signal was regained for endoglycosidase H N-deglycosylated His_6-Gpc-1 ΔHS and His_6-Gpc-1 N79Q,N116Q ΔHS, respectively, indicating that the N-linked glycans on Gpc-1 protected the core protein from irreversible denaturation (Figure 4A–C, triangles).

To further explore the unfolding of rhGpc-1 lacking N-linked glycans, we investigated the conformational stability of endoglycosidase H N-deglycosylated His_6-Gpc-1 ΔHS and His_6-Gpc-1 N79Q,N116Q ΔHS in urea. Denaturation by urea was monitored by measuring the ellipticity at 222 nm and the fluorescence intensity at 320 nm (with excitation at 280 nm) of rhGpc-1 that had been incubated in 0–9 M urea overnight. As for His_6-Gpc-1 ΔHS (Figure 5A,B), a single unfolding transition was observed for endoglycosidase H N-deglycosylated His_6-Gpc-1 ΔHS by both CD and fluorescence spectroscopy (Figure 5D,E). The data were well fitted using a two-state model from which values of $\Delta G^0_{H_2O}$ and m were obtained (eq 1). From these values, the urea concentration at 50% unfolding was calculated to be $C_M = \Delta G^0_{H_2O}/m$. The obtained values of C_M were close to identical for the secondary structure as monitored by CD spectroscopy (C_M = 5.51 M) and the tertiary structure as monitored by fluorescence spectroscopy (C_M = 5.49), indicating that CD and fluorescence report on the same process and that the unfolding equilibrium is a two-state process with no intermediates. In addition, urea denaturation of His_6-Gpc-1 N79Q,N116Q ΔHS showed a single unfolding transition when monitored by CD as well as fluorescence spectroscopy (Figure 5G,H), and the data were well fitted by a two-state model with calculated C_M = 5.37 M (CD) and C_M = 5.33 (fluorescence). The urea denaturation data were normalized using eq 2, and the baseline values obtained by fitting eq 1 to the data. The close overlap of normalized data obtained by CD and fluorescence spectroscopy (Figure 5C,F,I) indicates that the two methods report on the same transition and that there is concomitant denaturation of secondary and tertiary structure. Only minor differences were seen when comparing His_6-Gpc-1 ΔHS (C_M = 5.7 M), endoglycosidase H-treated His_6-Gpc-1 ΔHS (C_M = 5.5 M), and His_6-Gpc-1 N79Q,N116Q ΔHS (C_M = 5.3 M). Thus, all three proteins have similar stability toward urea denaturation and display highly cooperative unfolding indicative of a two-state process.

DISCUSSION

Glypicans exert their function by presenting HS chains to extracellular growth factors and thereby create morphogen gradients involved in pattern formation during development.[1] The two major classes of GAGs, HS and chondroitin sulfate (CS), are added to the same tetrasaccharide linker assembled on Ser residues in the GAG attachment domains of the core protein, which consist of repetitive motifs of the dipeptide Ser-Gly. Glypicans are exclusively substituted with HS, although it has been reported that Gpc-5 can exist as a hybrid containing both HS and CS.[16] The exact mechanism of GAG class determination has not been elucidated in detail, but expression of the GAG attachment domain of rat Gpc-1 without the N-terminal Cys-rich domain results in almost exclusive CS substitution, suggesting that the N-terminal part of the Gpc-1 core protein is involved in GAG class determination and preferential HS assembly.[17] Furthermore, it has been shown that mutations in parts of Gpc-3 that are not directly involved in HS attachment can nevertheless result in poor addition of HS to the core protein.[18] Together, these results indicate that a correctly folded N-terminal Cys-rich domain of Gpc-1 is required for efficient HS assembly.

In this work, we expressed Gpc-1 mutants for the two potential N-glycosylation sites at Asn-79 and Asn-116 and studied the effect on HS substitution, folding, and conformational stability of the core protein. Our results show that the two potential N-glycosylation sites in Gpc-1 are invariably occupied. The N-glycosylation mutants generated were exclusively substituted with HS, but with varying chain lengths, as demonstrated by cleavage of the GAG chains by HS lyase (Figure 2A,B). This result indicates correct folding of the protein despite disruption of N-glycosylation sites, since the expression of correctly folded Gpc-1 core protein is normally required for HS substitution. The N-glycosylation mutant His_6-Gpc-1 N79Q,N116Q, was expressed only as a high-molecular-weight HSPG, suggesting that the N-linked glycans on Gpc-1 are not involved in GAG class determination but may affect the amount of GAG substitution and also chain elongation. One explanation for this would be an affect at the level of protein expression, as it is known that the level of expression of the core protein correlates with the amount of HS substitution and elongation. Thus, when a low amount of Gpc-1 core protein is produced, the enzymes responsible for HS substitution and elongation will be present in excess and be able to process the available core proteins. In our experiments, the lowest expression was seen for His_6-Gpc-1 N79Q,N116Q, the mutant generating the largest Gpc-1 PG. It cannot, however, be excluded that the variation in protein expression is based on differences in gene expression due to selection of individual cell clones. On the other hand, according to our results, the amount of protein produced by the cell lines expressing Gpc-1 without glycosylation sites, His_6-Gpc-1 N79Q,N116Q ΔHS, correlated well with the expression levels obtained by tunicamycin treatment of cells expressing His_6-Gpc-1 ΔHS. The 50% reduction in protein yield observed indicates that disruption of N-glycosylation sites reduces Gpc-1 expression. Furthermore,

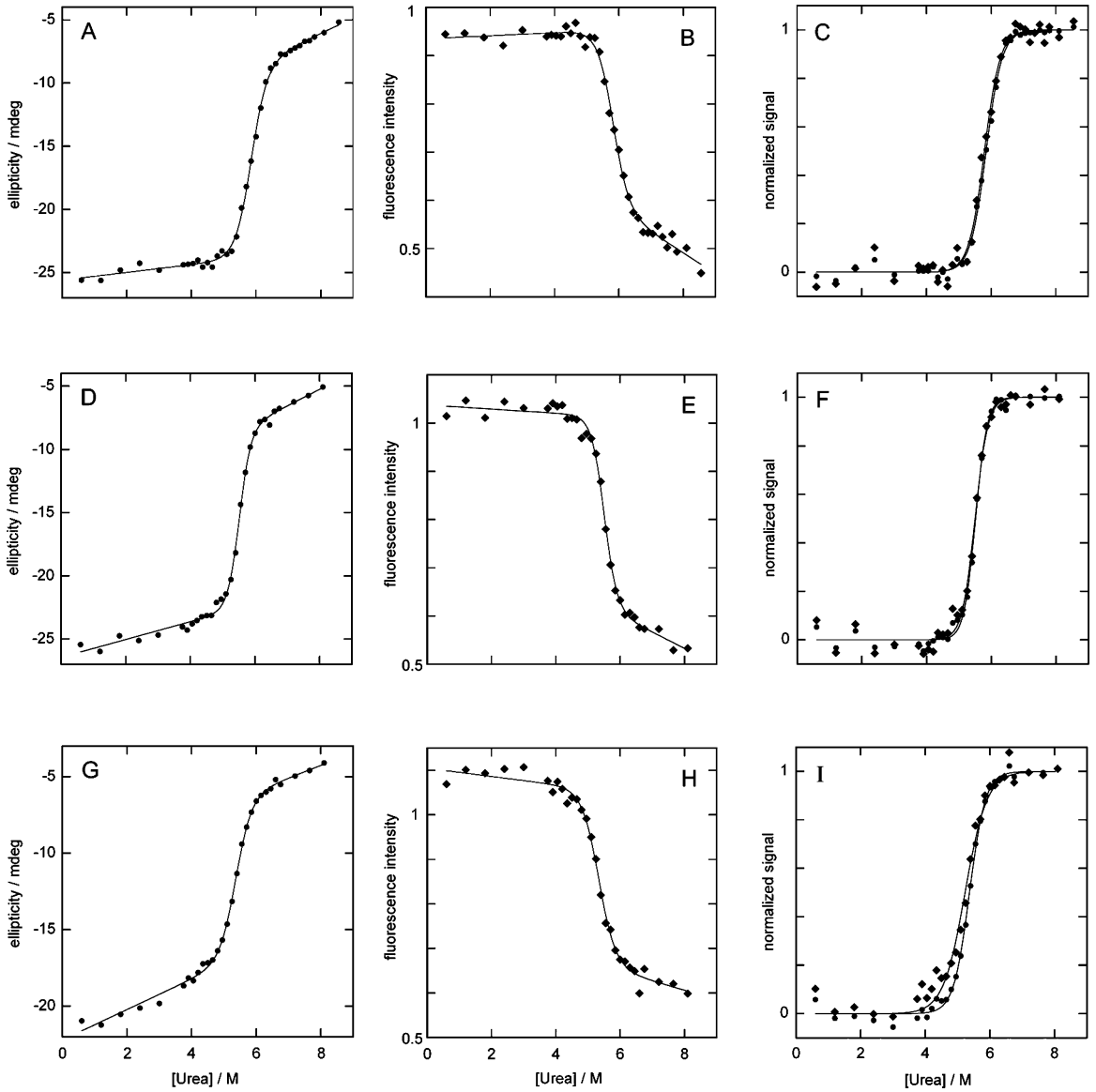

Figure 5. Urea denaturation of recombinant Gpc-1 with or without N-linked glycans monitored by far-UV CD and fluorescence. (A–I) His_6-Gpc-1 ΔHS (A, B, and C), endoglycosidase H-treated His_6-Gpc-1 ΔHS (D, E, and F), and His_6-Gpc-1 N79Q,N116Q ΔHS (G, H, and I) were incubated overnight in increasing concentrations of urea. Unfolding was monitored by ellipticity at 222 nm (filled circles, A, D, and G) or fluorescence at 320 nm (filled diamonds, B, E, and H). The CD and fluorescence data were fitted using a two-state model (solid lines). Data for the urea denaturation curves were normalized to apparent fraction unfolded (F_{app}): filled circles, normalized CD; filled diamonds, normalized fluorescence; solid lines, fitted curves (C, F, and I).

by comparing His_6-Gpc-1 ΔHS with wild-type His_6-Gpc-1 and also His_6-Gpc-1 N79Q,N116Q ΔHS with His_6-Gpc-1 N79Q,N116Q, we also detected that the presence of intact HS attachment sites lowers the level of protein expression. This contrasts with results shown for the PG decorin, where substitution with GAGs was found to increase the amount of protein produced when the three N-glycosylation sites in decorin had been removed by mutagenesis.[19] However, expression of decorin without intact glycosylation sites resulted in very poor protein expression, whereas expression of Gpc-1 was reduced by 50% either by tunicamycin treatment or mutagenesis. An alternative explanation for the difference in HS substitution would be an effect on the number of HS chains present in the different N-glycosylation mutant.

N-linked glycans play a role in the cotranslational folding of proteins in the ER.[12] N-linked glycans bind to the lectins calnexin and calreticulin, which act as molecular chaperones, keeping the unfolded proteins in the ER while the native structure is obtained. The oxidoreductase ERp57 is also involved in this process. In this way, N-linked glycans direct the folding events of proteins in the ER.[20] An analogous mechanism could be proposed for the folding of glypicans, but as we show here, Gpc-1 lacking glycosylation sites adopted a very similar conformational fold to that of Gpc-1 with intact N-glycosylation sites (Figure 4), suggesting that the folding of Gpc-1 can occur independently of calnexin/calreticulin. Gpc-2 and Gpc-6 belong to the same subfamily of glypicans as Gpc-1,[4] but they lack potential N-glycosylation sites (Supporting Information Figure S1), also indicating that folding of glypicans can occur independently of calnexin. Thus, it is likely that other molecular chaperones such as BiP and protein disulfide isomerase are able to promote the folding of glypicans in

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eukaryotic cells and that the N-linked glycans on glypicans exert local and not global effects on the folding of the protein.

By monitoring denaturation by heat and urea, we found that enzymatically deglycosylated Gpc-1 has a very similar stability and unfolding pathway to that of Gpc-1 with intact N-linked glycans (Figures 4 and 5). This is normally the case for deglycosylated proteins.[21] Furthermore, it has been shown that the first saccharide unit of the N-linked glycan confers most of the stabilization induced by the N-glycan chain,[22] suggesting that removal of N-linked glycans using endoglycosidase H may have little effect on the stability of the protein. Here, it was not possible to remove the N-linked glycans from native Gpc-1 by using PNGase F, indicating that the N-linked glycans on Gpc-1 are buried in or interact with the core protein. On the basis of structures in the Protein Data Bank in which the N-glycosylation sites were present, Petrescu et al.[23] estimated that the majority of N-glycan sites in proteins are found in convex or flat regions while 10% of the glycans are situated in deep narrow recesses, with one or two of the first glycan residues interacting with the protein. The N-linked glycans on Gpc-1 are most likely attached to the protein before the tertiary structure is adopted since they appear to be buried in the native protein, suggesting that the N-linked glycans on Gpc-1 have a role in cotranslational folding. However, we found here that it is possible to express Gpc-1 without any glycosylation and to obtain this mutant protein in large quantities. This protein appears to have a very similar fold to that of Gpc-1 with N-linked glycans (Figure 4). The thermostability of His_6-Gpc-1 N79Q,N116Q ΔHS (T_m reduced by 5 °C) was more affected compared to Gpc-1 in which the N-linked glycans had been removed by kifunensine/endoglycosidase H treatment (T_m reduced by 2 °C) from T_m 71 °C (Figure 4E). The single mutants His_6-Gpc-1 N79Q and His_6-Gpc-1N116Q, with enzymatically removed HS chains, behaved similarly as the double mutant His_6-Gpc-1 N79Q,N116Q ΔHS, indicating that lack of a single N-linked glycan was enough to destabilize the protein. The heat denaturation curve of the N-glycosylation mutants showed a shoulder between 45 and 60 °C. This shoulder was not present in the urea denaturation of His_6-Gpc-1 N79Q,N116Q ΔHS, which showed a single steep unfolding transition indicating a highly cooperative unfolding process (Figure 5G–I). The transition occurred at the same concentration for both secondary and tertiary structure, which is strong evidence that the unfolding equilibrium is a two-state process with no intermediates. This indicates that His_6-Gpc-1 N79Q,N116Q ΔHS is correctly folded. N-linked glycans often protect hydrophobic patches in proteins that would otherwise be negative for the solubility of the protein.[23] This would explain the different heat denaturation profiles of the N-glycosylation mutants compared to His_6-Gpc-1 ΔHS. The single GlcNAc residue left on the protein after cleavage with endoglycosidase H may be enough to retain this function of the N-linked glycans on Gpc-1, as very similar heat denaturation profiles were obtained for Gpc-1 ΔHS and endoglycosidase H-treated Gpc-1.

HSPGs sequester growth factors with low-affinity binding sites in their HS chains and present the growth factors to high-affinity receptors. However, recent reports using constructs expressing glypicans without HS have suggested a role also for the core protein itself in growth factor signaling. It is often desirable to remove the N-linked glycans on a protein prior to crystallization trials since such glycans are chemically and conformationally heterogeneous. The results presented in this paper indicate that the N-linked glycans on Gpc-1 can be removed by mutagenesis without compromising the folding of the protein. This is surprising since the N-glycosylation sites are invariably occupied. Disruption of both N-glycosylation sites resulted in lower protein yields, but it was still possible to obtain enough purified protein for structural studies. On the other hand, the kifunensine treatment of 293 cells producing His_6-Gpc-1 ΔHS rendered the protein sensitive to endoglycosidase H without reduced protein yield. Cleavage with endoglycosidase H was performed at pH 7.0 since Gpc-1 core protein is sensitive to low pH, but although this is not optimal for endoglycosidase H, only small amounts of enzyme were needed to remove the N-linked glycans from Gpc-1, making this the preferred method for removal of the N-linked glycans on Gpc-1 prior to crystallization.

Our results have implications for functional studies of glypicans, as the differential N-glycosylation on glypican core proteins is very likely to affect the amount of protein expressed and the length, and also possibly the fine structure, of the HS chains. Also, it has been shown that N-linked glycans can affect the subcellular localization of GPI-anchored proteins in polarized cells.[24] Apical sorting and subsequent endocytosis of Dally-like protein to the basolateral membrane is required for full-strength hedgehog signaling,[25] but the effect of N-linked glycans on subcellular localization has never been investigated. We are currently performing experiments to pursue this line of thinking.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1–S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

CD, circular dichroism; GAG, glycosaminoglycans; Gpc-1, glypican-1; GPI, glycosylphosphatidylinositol; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; PBS, phosphate-buffered saline; PG, proteoglycan; T_m, midpoint temperature; TEV protease, tobacco-etch virus protease; Tris, tris-(hydroxymethyl)aminomethane.

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