Glypican (heparan sulfate proteoglycan) is palmitoylated, deglycanated and reglycanated during recycling in skin fibroblasts

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Skin fibroblasts treated with brefeldin A produce a recycling variant of glypican (a glycosylphosphatidylinositol-anchored heparan-sulfate proteoglycan) that is resistant to inositol-specific phospholipase C and incorporates sulfate and glucosamine into heparan sulfate chains (Fransson, L.-A. et al., Glycobiology, 5, 407–415, 1995). We have now investigated structural modifications of recycling glypican, such as fatty acylation from [3H]palmitate, and degradation and assembly of heparan sulfate side chains. Most of the [3H]-radioactivity was recovered as lipid-like material after de-esterification. To distinguish between formation of heparan sulfate at vacant sites, elongation of existing chains or degradation followed by re-elongation of chain remnants, cells were pulse-labeled with [3H]glucosamine and then chase-labeled with [14C]glucosamine. Material isolated from the cells during the chase consisted of proteoglycan and mostly [3H]-labeled heparan-sulfate degradation products (molecular mass, 20–80 kDa) showing that the side chains were degraded during recycling. The degradation products were initially glucuronate-rich, but became more iduronate-rich with time. The glypic an proteoglycan formed during the chase was degraded either with alkali to release intact side chains or with heparinase to generate distally located chain fragments that were separated from the core protein, containing the proximally located, covalently attached chain remnants. All of the [14C]-radioactivity incorporated during the pulse was found in peripheral chain fragments, and the chains formed were not significantly longer than the original ones. We therefore conclude that newly made heparan-sulfate chains were neither made on vacant sites, nor by extension of existing chains but rather by re-elongation of degraded chain remnants. The remodeled chains made during recycling appeared to be more extensively modified than the original ones.

Key words: fatty acylation/glypican/heparan sulfate/recycling/reglycanation

Introduction

Proteoglycans are special forms of glycoproteins that are covalently substituted with linear and sulfated glycosaminoglycans (keratan sulfate, chondroitin sulfate, dermatan sulfate, heparan sulfate, or heparin or combinations thereof). They have a wide tissue distribution and occur in various forms of extracellular matrices, at cell surfaces and in intracellular granules (for reviews, see Kjellén and Lindahl, 1991; Bernfield et al., 1992; David, 1993; Heinegard and Oldberg, 1993). Proteoglycans are classified according to the characteristic features or properties of the core protein and can appear in many glycoforms giving rise to considerable structural variation and functional diversity. In general, the protein part determines the destination of the proteoglycan and interacts with other molecules at the final location. The glycan part provides the overall bulk properties as well as binding sites for other glycosaminoglycans and many types of proteins, including matrix proteins, plasma proteins, enzymes, anti-proteinases, growth factors, and cytokines.

Cultured human fibroblasts synthesize, deposit, and secrete a variety of proteoglycans and have been used extensively to investigate both their biosynthesis and functional properties (see, e.g., Carlstedt et al., 1983; Lories et al., 1987; Heremans et al., 1988; Schmidtchen et al., 1990a). Their plasma membrane-bound proteoglycans fall into two categories: those intercalated via membrane-spanning protein segments, for example, the syndecans, and those covalently linked to membrane lipids of the phosphatidylinositol (PtdIns)-type, so-called glypiated proteoglycans (for review, see Bernfield et al., 1992; Yanagishita and Hascall, 1992; David, 1993). Treatment of fibroblasts with PtdIns-specific phospholipase C releases a heparan-sulfate proteoglycan with a 60–70 kDa-core protein (David et al., 1990; Schmidtchen et al., 1990b) which, after molecular cloning of its cDNA, was given the name glypican (David et al., 1990).

Glycosyl-PtdIns-anchored membrane proteins are fairly common and include a variety of different protein families (for reviews, see Englund, 1993; Udenfriend and Kodukula, 1995). Proteoglycans linked in this manner include the above-mentioned glypican with a 64 kDa core protein and found in many different cell types (for review, see David, 1993) and K-glypican with a 57.5 kDa core protein (Watanabe et al., 1995) as well as other closely related forms, such as cerebroglycan (Kar thikeyan et al., 1992; Stipp et al., 1994), one species attached to muscle cells (Campos et al., 1993), another with a 39 kDa core protein isolated from adipocytes (Misra et al., 1994), and a developmentally regulated intestinal form, named OCI-5 (Filbus et al., 1995). The exact function of the lipid anchor is not clear. In polarized cells, it may serve to direct proteins and proteoglycans to the apical surface. In other cases, glypiated products may be directed and concentrated to specific membrane patches, called caveolae (Anderson, 1993). Biosynthesis and turn-over of glypican have been studied in granulosa cells (see Yanagishita, 1992; Yanagishita and Has call, 1992). In these cells, glypican and other heparan sulfate-proteoglycans are segregated and directed to separate degradation pathways. Glypican seems to be exclusively internalized and degraded. However, in a parathyroid cell line, Takeuchi et al. (1990) demonstrated recycling of heparan sulfate proteoglycan between the cell surface and an intracellular compart-
ment. Partial endoglycosidic degradation of heparan sulfate by endo-β-glucuronidase has been detected in many types of cells (for references, see Schmitzchen and Fransson, 1994).

The core proteins of proteoglycans are synthesized on membrane-bound ribosomes in the endoplasmic reticulum, and then transported to the Golgi, where glycan side chains are assembled. Mature proteoglycans are either secreted into the extracellular space or retained at the cell surface. By using brefeldin A to block transport from the endoplasmic reticulum to the Golgi, we have previously shown that most of the proteoglycans produced by skin fibroblasts are derived from newly synthesized core proteins (Fransson et al., 1992). However, a portion (~20%) of cell surface-bound heparan sulfate proteoglycan can be metabolically labeled with both radiosulfate and radioactive carbohydrate precursors in the presence of brefeldin A. In the presence of suramin, which blocks internalization and deglycanation of proteoglycans, skin fibroblasts accumulated a membrane-bound heparan sulfate proteoglycan with a 60–70 kDa core protein (Fransson et al., 1995). When both drugs were used simultaneously, no radiolabeled proteoglycan was detected suggesting that the radiolabeled proteoglycan was derived from resident cell-surface proteoglycan. After chemical biotinylation of cell-surface proteoglycan followed by metabolic radiosulphation, in continuously brefeldin A–treated cells, biotin-tagged radiolabeled proteoglycan was demonstrated, indicating the presence of recycling proteoglycan species. To determine the nature of the core protein, fibroblasts were pulse-labeled with [3H]leucine or [3H]inositol in the presence of suramin, followed by chase-labeling with [35S]sulfate in the presence of brefeldin A. A hydrophobic, glycosyl-Phosphatidylinositol-anchored, heparan sulfate proteoglycan with a 60–65 kDa core protein was obtained, indicating that it was glypican. However, the proteoglycan was resistant to digestion with Proteinase K, which could be due to fatty acylation of the inositol moiety.

In the present study we have investigated whether the recycling glypican variant can be fatty acylated, and whether the heparan sulfate side chains are built on vacant sites, if existing chains are directly elongated or if they are degraded and re-elongated.

Results

Recycling glypican incorporates [3H]palmitate and is unaffected by monensin

Skin fibroblasts were incubated with [3H]palmitate and [35S]sulfate in the presence of brefeldin A and polyanionic macromolecular material was isolated from the detergent extract of the cells (see Figure 1 caption). [3H]Radioactivity co-chromatographed, in the presence of detergent, with the [35S]proteoglycan material both on Superose 6 (Figure 1A) and on Mono Q (Figure 1B) in the positions expected for glypican (Fransson et al., 1992, 1995). [3H] and [35S]proteoglycan was also bound to octyl-Sepharose and was eluted with detergent (Figure 1C). Proteoglycan material displaced from octyl-Sepharose by de-esterified with alkali (two different methods were used) and subjected to lipid extraction. The organic phase contained 70–95% of the [3H]-radioactivity but only 2–4% of the [35S]-radioactivity. Only 4% of the [3H]- and 1% of the [35S]-radioactivity was found in the organic phase after extraction of untreated proteoglycan. Incubation of the cells with radioactive precursors together with pyruvate and nonessential amino acids (Masterson and Magee, 1992) increased incorporation of radiolabeled proteoglycan up to 2-fold, but did not otherwise affect the results (data not shown).

Fibroblasts were also incubated with [35S]sulfate in the presence of brefeldin A and monensin (3 x 10^-6 M), and hydrophobic proteoglycans were isolated on octyl-Sepharose. The yield of radiolabeled proteoglycan was unaffected by monensin treatment. In cells not treated with brefeldin A, monensin markedly inhibited overall proteoglycan production (results not shown).
The heparan sulfate side chains of recycling glypican are highly modified

Previous studies have shown that the side chains of recycling glypican are unusually long and highly charged (Fransson et al., 1992, 1995). In the present study, we examined the glycan structure by digestion with heparitinase which cleaves hexosaminidic bonds to D-glucuronic acid (GlcA) in the un- and low-modified regions (Figure 2A). Gel-filtration chromatography of the degradation products afforded both disaccharides and a series of higher oligosaccharides (Figure 2B). Hence, the GlcA-containing repeats occurred both in clusters and in alternating or mixed arrangements with iduronic acid (IdoA)-containing repeats. The nonsulfated and sulfated disaccharides were partly resolved (see also Lindblom and Fransson, 1990) indicating that a minor proportion of the disaccharides were derived from repeats that contained GlcA and N-sulfamidoglucosamine (GlcNSO₃). The total proportion of GlcA-containing repeats was estimated to 50%.

Recycling glypican is deglycanated

To account for the incorporation of [³H]glucosamine into recycling glypican in the presence of brefeldin A (Fransson et al., 1992, 1995), there are three principally different possibilities (Figure 3A). When cell surface-residing glypicans are endocytozed and returned to the Golgi, (1) new heparan-sulfate chains are built on vacant sites, or (2) existing chains are directly elongated, or (3) existing chains are degraded and core-protein-attached remnants are then used as primers for re-elongation. To distinguish between these possibilities we pulse-labeled cells with [³H]glucosamine in the presence of suramin (or in the presence of brefeldin A, or with no drug present). We then chase-labeled the cells in medium containing [¹⁴C]glucosamine and brefeldin A, for various periods of time (Figure 3A). Material extracted from the cells (and recovered from the media) was separated into proteoglycan and heparan sulfate oligosaccharides. The structure of the latter was examined by enzymatic or chemical degradations. The heparan sulfate side chains of the proteoglycan were cleaved by heparitinase at sulfated IdoA, and sulfated GlcA if present (Yamada et al., 1995), in the highly modified regions (Figure 3B). This yielded core protein with covalently attached remnants and heparan-sulfate chain-fragments (IV in Figure 3A). This approach has been successfully employed previously (Lindblom et al., 1991). We finally examined whether the [¹⁴C]glucosamine that was incorporated during the chase was preferentially found in the

Fig. 2. (A) Degradation of glypican-derived heparan sulfate by heparitinase and (B) gel-filtration chromatography of the products on Biogel P-6. Heparan sulfate side chains usually display a periodic, complex copolymeric disaccharide pattern characterized by a block structure composed of extended unsulfated regions containing GlcA and largely N-acetylated GlcN (empty circles in A) interrupted by more sulfated and modified regions containing progressively increasing proportions of N-sulfated GlcN and IdoA as well as of ester-sulfate in both sugars (progressively shaded circles in A). The disaccharides near the linkage-region to the core protein are usually unmodified. Heparitinase cleaves in the unmodified and moderately modified regions (principally as indicated in the blow-up in A). Although heparitinase can also cleave bonds between GlcNAc and IdoA in an artificial substrate, such as totally desulfated and re-N-acetylated/re-N-sulfated heparin (Desai et al., 1993; Fransson L.-Å. and Havemark, B., unpublished observations), such linkages do not normally arise in biologically produced glycan chains, owing to the specificity of the uronosyl 5-epimerase (Jacobsson et al., 1984). Oligosaccharides generated by treatment with heparitinase are resolved according to size by gel-filtration chromatography (B). In the actual experiment, confluent cultures were incubated with [³H]glucosamine and [³⁵S]SO₄ in the presence of brefeldin A, and the cell-associated proteoglycan pool was isolated as described in Materials and methods. Heparan sulfate side chains were released by treatment with alkaline borohydride and recovered by chromatography on DEAE-cellulose. Finally the chains were digested exhaustively with heparitinase and chromatographed on Biogel P-6. The elution positions of disaccharide (2), tetrasaccharide (4), and higher saccharides (6–20) are indicated. As heparitinase cleaves bonds to GlcA, the yield (as [³H]) of the various saccharides was used to calculate the content of GlcA in heparan sulfate according to the formula shown in B. A is the amount of [³H] in a particular saccharide, and n is the size.
distally located, heparinase-released fragments, or in the proximally located, core protein-bound remnants, or both.

Polyanionic material recovered from cell extracts after pulse-labeling with \[^{3}H\]glucosamine in the presence of suramin, followed by chase-labeling with \[^{14}C\]glucosamine in the presence of brefeldin A, was subjected to gel-filtration chromatography (Figure 4). It consisted of macromolecules, including proteoglycans (PG), that eluted in the void volume, as well as lower molecular mass degradation-products, that is, heparan sulfate oligosaccharides (OS) and glycan-chains ranging in size from approximately 20 to 80 kDa. During the first 4 h of chase the degradation products were largely \[^{3}H\]labeled, but after 20 h of chase \[^{14}C\]labeled material began to appear in substantial amounts. The yield of \[^{3}H\]labeled degradation products reached a maximum after 4 h (Table I). \[^{3}H\]Labeled degradation products were also obtained when the chase followed a pulse carried out in the presence of brefeldin A or in the absence of drugs. The yield of degradation-products obtained after preincubation with suramin was approximately twice as high as in the other two cases, which gave similar results (data not shown). In general, most of the degradation products remained associated with the cells, but material released into the medium increased slowly with time (Table I).

The structural features of the heparan sulfate degradation products were examined by gel-filtration chromatography after enzymatic or chemical degradations (Figures 5, 6). The fragments released at early time points of the chase were slightly larger than those obtained later in the chase period (solid line in Figure 5). Furthermore, their sensitivity to heparitinase was also greater at early time points (dotted line in Figure 5) in keeping with a high proportion of GlcA-containing repeats in the released material. At all time points, the effect of heparinase (dashed line in Figure 5) was only marginal, indicating a low content of sulfated uronic acids. The degradation products obtained after a 20-48 h chase period were largely resistant to both heparitinase and heparinase (a 20 h sample is shown in Figure 5C), suggesting that non-sulfated IdoA was common in these products. As IdoA should occur in combination with GlcNSO₃, we treated a 48 h sample with nitrous acid at pH 1.5 and examined the products by gel-filtration chromatography (Figure 6). It is seen that extensive degradation took place and the amount of GlcNSO₃ was estimated to 51%.

**Recycling glypican is reglycanated**

The radio-labeled polyanionic macromolecules extracted from the cells after pulse-labeling with \[^{3}H\]glucosamine in the pres-
Reglycanation of recycling glypican

e of suramin followed by chase-labeling with [\(^{14}\)C]glucosamine in the presence of brefeldin A, were examined by ion-exchange FPLC on Mono Q (Figure 7). In this step, hyaluronan (HA) was removed and the proteoglycan (PG) pool was resolved into heparan sulfate (HS)-containing and dermatan sulfate (DS)-containing species. The latter species gradually disappeared during the chase (Figure 7A–C) and after 48 h only heparan sulfate proteoglycan remained (Figure 7D). By that time its \(^{14}\)C content had increased to approx. twice that of \(^{3}\)H. These results (Figures 4–7) demonstrate that recycling glypican releases heparan sulfate chain fragments and oligosaccharides and that new heparan sulfate chains are added. These could be entirely new chains built on vacant sites or, alternatively, extensions of existing chains or of core-protein-attached remnants (see Figure 3A).

**New heparan sulfate chains are extensions of core-protein-attached remnants**

The \([^{3}\)H,\(^{14}\)C]-labeled glypican isolated (Figure 7D) after pulse-labeling with \([^{3}\)H]glucosamine in the presence of suramin followed by chase-labeling with \([^{14}\)C]glucosamine in the presence of brefeldin A, was degraded with heparinase and the products, that is, nonhydrophobic chain fragments and hydrophobic core-protein with covalently attached remnants, were separated using octyl-Sepharose (Lindblom et al., 1991). It is seen (Figure 8B) that all of the \(^{14}\)C-radioactivity appeared in the nonhydrophobic fraction together with approx. half of the \(^{3}\)H-radioactivity. Hence, the remnants on the core-protein were exclusively \(^{3}\)H-labeled. These results indicate that chain-elongation on vacant sites during the chase is unlikely. In such a case, \(^{14}\)C-labeled remnants should have been found in the core-protein preparation after heparinase degradation (see Figure 3A, alternative I).

To distinguish between the two other possibilities (alternatives II and III in Figure 3A), the size of the heparan sulfate side chains and their content of \(^{3}\)H- and \(^{14}\)C-radioactivity was

Table I. Yield of \(^{3}\)Hglucosamine-labeled heparan sulfate oligosaccharides (OS) released during chase in the presence of brefeldin A

<table>
<thead>
<tr>
<th>Source</th>
<th>Time (h)</th>
<th>Amount (dpm.)</th>
</tr>
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<tbody>
<tr>
<td>Cell extract</td>
<td>1</td>
<td>120,000</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>160,000</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>110,000</td>
</tr>
<tr>
<td>Medium</td>
<td>1</td>
<td>31,000</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>53,000</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>72,000</td>
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</table>

For details, see Figure 4 caption.

Fig. 4. Gel-filtration chromatography on Superose 6 of cell-derived proteoglycans and degradation products obtained after pulse-labeling with \([^{3}\)H]glucosamine in the presence of suramin followed by chase-labeling with \([^{14}\)C]glucosamine in the presence of brefeldin A for (A) 1 h, (B) 4 h, (C) 20 h, and (D) 48 h. Cells grown to confluence in 25-cm\(^2\) dishes were incubated with 20 \(\mu\)Ci/ml \([^{3}\)H]glucosamine and 0.2 mM suramin in regular medium at 37°C for 24 h. The medium was then removed and changed to fresh medium containing 50 \(\mu\)Ci/ml \([^{14}\)C]glucosamine and brefeldin A (10 \(\mu\)g/ml). After further incubation for 1, 4, 20, and 48 h the media were collected and the cells were washed and extracted with Triton X-100. Polyanionic molecules were recovered from media and cell extracts by ion exchange chromatography on Q-Sepharose and DEAE-cellulose, respectively, and finally subjected to gel-filtration chromatography. The results obtained with the cell-derived material are shown (see also Table I). One excluded proteoglycan (PG) pool and one, partially included, oligosaccharide (OS) pool were collected in each case (see bar). The proteoglycan pools were further purified and fractionated by ion exchange FPLC on MonoQ (see Figure 7). \(V_v\), void volume or volume of mobile phase; \(V_t\), total volume of gel. The elution positions of heparan sulfate (HS) standards (80, 45, and 17 kDa) are indicated.

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Fig. 5. Gel-filtration chromatography on Superose 6 of untreated (solid lines), heparinase-treated (dashed lines), and heparitinase-treated (dotted lines) heparan sulfate degradation products obtained after pulse-labeling with $[^3]$H]glucosamine in the presence of suramin followed by chase-labeling with $[^14]$C]glucosamine in the presence of brefeldin A for 48 h. The degradation products (pool OS) collected in Figure 4D were recovered by ion exchange chromatography on Q-Sepharose and subjected to enzymic degradation as described in Materials and methods. The content of GlcNSO$_3$ was estimated (using $[^3]$H-radioactivity) by the same calculation as in Figure 2.

determined. As shown in Figure 9A the size of the side chains (released from glypican by alkaline elimination) was ~100 kDa. The $[^14]$C-radioactivity was not particularly enriched in the longest chains making alternative II in Figure 3A rather unlikely.

The peripheral heparan sulfate fragments released from glypican by heparinase and separated from the core-protein (Figure 3A, IV) by hydrophobic interaction chromatography (Figure 8B, fractions 1–2) contained two distinct size pools (Figure 9B), one ~45 kDa with a high proportion of $[^14]$C, and another ~5–10 kDa with approximately equal amounts of the two isotopes. These results are best explained as follows. Some of the side chains in recycling glypican (see panel I in Figure 9) were only marginally degraded, whereas others were extensively eroded. In addition, heparinase-sensitive sites are probably more common in remodeled chains and also located closer to the core protein. Hence, long, heparinase-released fragments could be relatively enriched with $[^14]$C-radioactivity compared to shorter ones (see panels I and II in Figure 9).

To determine if the marginally and the extensively degraded side chains were on separate or the same glypican molecules (see panel I in Figure 9), the core protein preparation recovered after heparinase degradation (Figure 8B, fractions 7–8) was subjected to gel-filtration chromatography (Figure 9C). A broad, but uniform peak was obtained that eluted earlier than the largest chain fragments (Figure 9B) indicating that both long and short side chains were on the same core protein (see panel II of Figure 9). Accordingly, the core protein-bound chains and chain remnants that were released by alkaline elimination and rechromatographed (Figure 9D) contained two size pools, one with 45–100 kDa and another with 5–45 kDa (see also panel IV in Figure 9).

Discussion

Proteins destined to become linked to glycosyl-PtdIns are made as ordinary secretory proteins and directed to the endoplasmic reticulum via an N-terminal signal peptide (see Figure 10, site I). After removal of the signal peptide a second hydrophobic C-terminal peptide serves as recognition site for a transamidase
Fig. 7. Ion-exchange FPLC on Mono Q of the proteoglycan pools obtained after pulse-labeling with $[^{3}H]$glucosamine in the presence of suramin followed by chase-labeling with $[^{14}C]$glucosamine in the presence of brefeldin A for (A) 1 h, (B) 4 h, (C) 20 h, and (D) 48 h. The void volume fractions (pool PG) collected in Figure 4A–D were subjected to ion exchange FPLC on Mono Q as described in Materials and methods. Elution was performed with a linear gradient from 0.3 M NaCl (10 min) to 1.2 M NaCl (70 min). The peaks obtained correspond to hyaluronan (HA), heparan sulfate proteoglycan (HS), and dermatan sulfate proteoglycan (DS). The heparan sulfate proteoglycan obtained in (D) was pooled as indicated by the bar.

Fig. 8. Hydrophobic interaction chromatography on octyl-Sepharose of (A) untreated and (B) heparinase-treated glypican obtained after pulse-labeling with $[^{3}H]$glucosamine in the presence of suramin followed by chase-labeling with $[^{14}C]$glucosamine in the presence of brefeldin A for 48 h. The heparan sulfate proteoglycan pool obtained in Figure 7D was subjected to chromatography before and after digestion with heparinase as described in Materials and methods.

(see Udenfriend and Kodukula, 1995) that removes the C-terminal peptide by proteolytic cleavage and transfers the C-terminal end of the protein to an ethanolamine moiety of a preformed glycosyl-PtdIns anchor (Site II in Figure 10). The glycerol in PtdIns is usually esterified with fatty acyl groups, but ether-linked alkyl groups have also been found. The inositol moiety can be acylated, usually with palmitate, yielding an anchor with three hydrocarbon chains. Acylation of the inositol moiety confers resistance to PtdIns-specific phospholipase C. Remodeling of the lipid-anchor by exchange of acyl groups during transport to the cell surface has also been proposed (see Englund, 1993). More recently, results with glycosyl-PtdIns-linked placental alkaline phosphatase (Wong and Low, 1994), indicate that the newly synthesized phosphatase protein is phospholipase C-sensitive but acquires resistance as it matures. Inositol acylation may thus also take place after transport to the Golgi.

Glycosyl-PtdIns-anchored proteins destined to become proteoglycans (such as glypican) are substituted with glycosaminoglycan side chains in the trans-Golgi. After addition of heparan-sulfate side chains and transport to the cell-surface (site III in Figure 10) glypicanc proteoglycans may remain at the cell-surface, or they may be shed into the medium or internalized by endocytosis. Shedding may be initiated by proteolysis or by cleavage of the lipid-anchor by PtdIns-specific phospholipase C (see David, 1993). In the latter case, it must be preceded by
Fig. 9. Gel-filtration chromatography on Superose 6 of (A, panel I) intact, alkali-released heparan sulfate chains from glypican, (B, panel II) heparinase-released peripheral chain fragments, (C, panel III) glypican core-protein with covalently attached chain remnants, and (D, panel IV) alkali-released chain remnants. [3H/14C]Glucosamine-labeled glypican was obtained by pulse-labeling with [3H]glucosamine in the presence of suramin followed by chase-labeling with [14C]glucosamine in the presence of brefeldin A for 48 h (see Figure 8D). Heparan sulfate was released from the glypican core-protein by alkaline cleavage and recovered by chromatography on DEAE-cellulose as described in Materials and methods. The glypican preparation was treated with heparinase (see panel I) and peripheral chain-fragments (see panel II) were separated from the core-protein with its covalently attached remnants (see panel III) by hydrophobic interaction chromatography on octyl-Sepharose as shown in Figure 8B. The core-protein-attached chain remnants were released by alkaline cleavage (see panel IV) and recovered by chromatography on Q-Sepharose. The elution position of glypican proteoglycan (HSPG) is indicated. For further details, see Figure 4 caption.

Fig. 10. Model for the biosynthesis, secretion, endocytosis, and recycling of glypican. Site I: Biosynthesis of the core protein in the rough endoplasmic reticulum (RER), proteolytic trimming and transfer to the preformed glycosyl-Ptdlns-anchor, perhaps containing both glycerol- and inositol-linked hydrocarbon (acyl and/or alkyl) chains, followed by transport to the Golgi complex. Site II: The trans-Golgi compartment where the heparan sulfate side chains are assembled, followed by secretion to the cell-surface. Site IIIa: Cell-surface bound glypican which may be deacylated at the inositol moiety and remain stationary at the surface (Site IIIb). It is postulated that deacylated glypican (Site IIIa) is internalized by endocytosis. Site IV: Endosome with partially deglycansated glypican that is recycled back to the trans-Golgi (Site II) where new heparan sulfate chains are rebuilt on remaining stubs.

Fig. 11. Model for the biosynthesis, secretion, endocytosis, and recycling of glypican. Site I: Biosynthesis of the core protein in the rough endoplasmic reticulum (RER), proteolytic trimming and transfer to the preformed glycosyl-Ptdlns-anchor, perhaps containing both glycerol- and inositol-linked hydrocarbon (acyl and/or alkyl) chains, followed by transport to the Golgi complex. Site II: The trans-Golgi compartment where the heparan sulfate side chains are assembled, followed by secretion to the cell-surface. Site IIIa: Cell-surface bound glypican which may be deacylated at the inositol moiety and remain stationary at the surface (Site IIIb). It is postulated that nondeacylated glypican (Site IIIa) is internalized by endocytosis. Site IV: Endosome with partially deglycansated glypican that is recycled back to the trans-Golgi (Site II) where new heparan sulfate chains are rebuilt on remaining stubs.

IV-II in Figure 10) possibly via acyl-exchange. Monensin had no effect on [35S]sulfate-incorporation into recycling glypican suggesting that endocytoxed glypican returns to the trans-Golgi compartment. The present results also show that, concomitant with internalization, either at the cell-surface or in endosomes (Site IV in Figure 10), the heparan-sulfate side chains of glypican are partially degraded. The fate of the released oligosaccharides is unknown at present, but the glypican core protein with its chain remnants return to the Golgi (Site II in Figure 10) where the remnants are used as primers for re-elongation. The recycling variant probably represents a minor fraction (approximately one-third) of the glypican molecules in fibroblasts (Fransson et al., 1995). As we have used inhibitors of cellular transport and/or degradation, an otherwise minor pathway may have been augmented. It may also have generated unusually long side chains in glypican. To investigate recycling of glypican in untreated cells, especially cytochemically, antibodies against the glypican core protein must be raised. Such developments are in progress.
The side chains derived from a pool of glypican-like heparan sulfate proteoglycans (dominated by species with ~70 kDa core proteins) produced by untreated skin fibroblasts were analyzed previously (Schmidtchen and Fransson, 1992). These side chains, which had an average molecular mass of ~50 kDa, contained ~70% GlcA, the majority of which were in extended low- or unmodified segments (see also Figure 3B). The amount of sulfated uronic acids was ~10%, the majority of which were in single repeats, probably clustered together with other types of IdoA-containing and highly modified repeats (see Figure 3B). In general, most types of heparan sulfate side chains from fibroblast proteoglycans appear to have very similar molecular design (Turnbull and Gallagher, 1991; Schmidtchen and Fransson, 1992). In contrast, the heparan sulfate side chains of the recycling glypican variant produced in the presence of brefeldin A were different in two ways: they were longer (over 100 kDa) and they contained a somewhat higher proportion of modified repeats (only 50% GlcA). If the recycling glypican constitutes a minor proportion of cell-surface attached heparan sulfate proteoglycans, these features may have been unnoticed.

As heparan sulfates generally have a low content of sulfated uronic acids, treatment with heparinase generates relatively large chain fragments (Lindblom et al., 1991; Turnbull and Gallagher, 1991; Schmidtchen and Fransson, 1992). The side chains of recycling glypican generated several different size pools of both free oligosaccharides and core-protein-bound remnants after heparinase treatment. The free oligosaccharides included one pool of small size (5-10 kDa) and one pool of larger fragments (45 kDa and above). The [3H]/[14C] ratios of these oligosaccharides were different, suggesting that the 45 kDa fragments were derived from segments largely synthesized during recycling. The core-protein-bound remnants, which were exclusively [3H]-labeled, included one pool of very large chains (near 100 kDa), almost as large as the intact chains, and one pool of smaller material (5-45 kDa). Taken together these results indicate that some chains remained largely intact during recycling, whereas others were extensively degraded and re-elongated.

The endogenously generated oligosaccharide fragments obtained at early time points of the chase had a relatively high content of GlcA, and became more modified with time, suggesting that the machinery for glycan assembly on de novo synthesized core protein precursors behaved differently from that used by the recycling ones. Heparan sulfate oligosaccharides of similar size (7-10 kDa), composition (high in GlcA), and molecular design (sulfated uronic acids at the periphery) were also endogenously produced in fibroblasts not treated with brefeldin A or suramin (Schmidtchen and Fransson, 1992). As the yield of such oligosaccharides was similar in the absence or presence of brefeldin A, it appears likely that it is mainly glypican side chains that are subject to degradation and re-elongation.

Return of cell-surface glycoproteins to compartments of the secretory pathway has been described previously (Litvinov and Hilken's, 1993; Volz et al., 1995, and references therein). The compartments that receive material from the endocytic pathway are usually the trans-Golgi or the trans-Golgi network. Resalivation of desialylated glycoproteins or sialylation at vacant sites have been demonstrated. The present results show that also complex remodeling, involving both glucosaminyl-, glucuronyl-, and sulfate-transfer as well as C-5 epimerization of GlcA to IdoA, can take place. The exact location of the various enzymes involved in heparan sulfate chain assembly is not known, but they are most likely concentrated to the distal compartments of the Golgi complex. Recycling of glypican through these compartments provides an opportunity for continual alteration in the molecular design of heparan sulfate. In this way cells can rapidly change their binding patterns vis-a-vis extracellular heparan sulfate-binding cytokines, growth factors, and endoheparanases.

Materials and methods

Materials

Radiolabeled heparan sulfate chains and chain fragments with known molecular size were obtained as described (Schmidtchen and Fransson, 1994). Cell culture media were from NordVacc, Sweden and the enzymes used were heparinase (EC 4.2.2.7) and heparitinase (EC 4.2.2.8) from Seikagaku Corp., Tokyo, Japan (corresponding enzymes supplied by Sigma are called heparinase I and III, respectively). Na2[15SO4] (1310 Ci/mmol), t[6-3H]glucosamine hydrochloride (20-40 Ci/mmol), [3-14C]glucosamine hydrochloride (200 Ci/mmol), and [9,10-3H]palmitic acid (40-60 Ci/mmol) were purchased from the Radiological Centre, Amersham, United Kingdom. The prepacked columns and column media were Superose 6 HR 10/30, MonoQ HR 5/5, octyl-Sepharose CL-4B, Q-Sepharose Fast Flow (all from Pharmacia-LKB, Sweden), Bio-Gel P-4 (Bio-Rad), and DE-53 DEAE-cellulose (Whatman). Special chemicals were brefeldin A and monensin (Sigma) and suramin (Bayer). Other chemicals were of analytical grade.

Cell culture and radiolabeling

Fibroblasts from human embryonic skin were grown as monolayers in plastic dishes using Eagle's minimal essential medium with Earle's salts supplemented with 10% (v/v) donor calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Confluent cultures between passages 5 and 15 were used in the experiments. Incorporation of [3H]glucosamine was performed in regular medium (see above). [35S]sulfate and [3H]palmitate/ [35S]sulfate in sulfate-deficient medium containing 1% (v/v) donor calf serum (see captions to the appropriate figures). Cells were preincubated in labeling medium for at least 1 h. This was then replaced with fresh medium containing radioisotope-labeled precursors (0.2 ml medium/cm² of dish surface). For further information, see Fransson et al. (1995).

Extraction and isolation of radiolabeled proteoglycans

The methods have been described in detail previously (Schmidtchen et al., 1990a,b; Schmidtchen and Fransson, 1993; Fransson et al., 1995) and include the following steps: (1) collection of media followed by brief extraction of the cells with Triton X-100 in the presence of inhibitors of serine-, thiol-, and metalloproteinases; (2) isolation of medium- and cell-derived polymeric material (hyaluronan, proteoglycans, glycans, and glycan-fragments) by binding to DEAE-cellulose; (3) separation of hyaluronan and proteoglycans from glycan chain fragments by gel-filtration chromatography; (4) separation of hyaluronic, heparan sulfate-, and chondroitin/dermatan sulfate proteoglycans by ion exchange chromatography; and (5) purification of membrane-bound proteoglycans by hydrophobic interaction chromatography. Concentration of proteoglycan solutions (fortified with dextran as carrier) was achieved either by passage over 0.2 ml columns of DEAE-cellulose or Q-Sepharose, or by precipitation with ethanol from NaOAc-containing solutions.

Degradation methods

Heparan sulfate chains were released from the proteoglycan core protein by treatment with alkaline borohydride (0.5 M NaOH, 0.1 M NaBH₄ at room temperature overnight). Enzymatic cleavage of heparan sulfate chains by heparinase or heparitinase was performed as described (Schmidtchen and Fransson, 1992, 1994; Fransson et al., 1995). Deaminative cleavage (at pH 1.5) of GlcNSO₃-GlcA/IdoA bonds in heparan sulfate was performed as described by Shively and Conrad (1976).

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Separation methods

Gel-filtration chromatography was performed in the FPLC mode on Superose 6 or in the standard mode on Bio-Gel P-6, ion-exchange chromatography in the FPLC mode on MonoQ, and hydrophobic interaction chromatography on octyl-Sepharose as described previously (Lindblom et al., 1989; Schmidchen and Fransson, 1992, 1993). Aliquots of the fractions were analyzed for radioactivity by liquid scintillation.

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Abbreviations

GlcA, α-glucuronic acid; GlcNSO₃, N-sulfamido glucosamine; IdoA, L-iduronic acid; PtdIns, phosphatidylinositol.

References


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