Heparan Sulfate Expression in Polarized Epithelial Cells: The Apical Sorting of Glypican (GPI-anchored Proteoglycan) Is Inversely Related to Its Heparan Sulfate Content

Griet Mertens, Bernadette Van der Schueren, Herman van den Berghe, and Guido David

Center for Human Genetics, University of Leuven, and Flanders Institute for Biotechnology, Leuven, Belgium

Abstract. Several processes that occur in the luminal compartments of the tissues are modulated by heparin-like polysaccharides. To identify proteins responsible for the expression of heparan sulfate at the apex of polarized cells, we investigated the polarity of the expression of the cell surface heparan sulfate proteoglycans in CaCo-2 cells. Domain-specific biotinylation of the apical and basolateral membranes of these cells identified glypican, a GPI-linked heparan sulfate proteoglycan, as the major source of apical heparan sulfate. Yet, most of this proteoglycan was expressed at the basolateral surface, an unexpected finding for a glypiated protein. Metabolic labeling and chase experiments indicated that sorting mechanisms, rather than differential turnover, accounted for this bipolar expression of glypican. Chlorate treatment did not affect the polarity of the expression of glypican in CaCo-2 cells, and transfectant MDCK cells expressed wild-type glypican and a syndecan-4/glypican chimera also in an essentially unpolarized fashion. Yet, complete removal of the heparan sulfate glycanation sites from the glypican core protein resulted in the nearly exclusive apical targeting of glypican in the transfectants, whereas two- and one-chain mutant forms had intermediate distributions. These results indicate that glypican accounts for the expression of apical heparan sulfate, but that glycanation of the core protein antagonizes the activity of the apical sorting signal conveyed by the GPI anchor of this proteoglycan. A possible implication of these findings is that heparan sulfate glycanation may be a determinant of the subcellular expression of glypican. Alternatively, inverse glycanation–apical sorting relationships in glypican may insure near constant deliveries of HS to the apical compartment, or "active" GPI-mediated entry of heparan sulfate into apical membrane compartments may require the overriding of this antagonizing effect of the heparan sulfate chains.

THE heparan sulfate proteoglycans (HSPGs)[1] of the cell surface bind a variety of ligands. The activities of these ligands and the functional consequences of their interactions with heparin-like polysaccharides implicate the heparan sulfate proteoglycans in mechanisms of cell adhesion and recruitment, cell differentiation and proliferation, and in the control of proteolytic and lipolytic pathways (reviewed by Bernfield et al., 1992; David, 1993). Fibroblast growth factors and vascular endothelial growth factor, for example, depend on heparan sulfate (HS) for binding to their cognate receptors and for eliciting a biological response from this receptor occupancy (Yayon et al., 1991; Rapraeger et al., 1991; Gitay-Goren et al., 1992; Spivak-Kroizman et al., 1994). There is also evidence that glycosaminoglycan-bound chemokines recruit T cells from the circulation (Tanaka et al., 1993), that the binding of antithrombin III to endothelial HS accelerates the interaction of this proteinase inhibitor with proteases of the coagulation cascade (Bourin and Lindahl, 1993), and that lipoprotein lipase is transcytosed through endothelia and anchored to the endothelial membrane by mechanisms that involve binding to cell surface–associated HS (Saxena et al., 1991*a*,*b*).

Several distinct proteins have been identified as major carriers of the HS that is associated with the surface of mammalian cells. Four of these, the syndecans, are transmembrane proteins and are grouped as a family because of striking similarities in domain structure and shared sequence motifs. The membrane-spanning and cytoplasmic domains of the syndecans, in particular, show significant sequence similarity and have been strongly conserved during evolution (Bernfield et al., 1992). Their mode of association with the cell surface contrasts with that of three other proteoglycans, known as glypican (David et al.,

Address for correspondence to Guido David, Center for Human Genetics, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. Ph.: (32)16-345863. Fax: (32) 16-345997. E-mail: Guido.David@med.kuleuven.ac.be

1. *Abbreviations used in this paper*: CEA, carcinoembryonic antigen; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; OG, octylglucoside; TX-100, Triton X-100.

1990), cerebroglycan (Stipp et al., 1994), and K-glypican (Watanabe and Yamaguchi, 1995), that are linked to the cell surface via glycosyl phosphatidylinositol (GPI). Together with another candidate member, the protein encoded by the OCI-5 transcript (Filmus et al., 1988), these proteins define a second family of integral membrane HSPGs, characterized by evolutionarily conserved protein cores of similar sizes that share the GPI anchorage mechanism, a unique cysteine motif that may be involved in the stabilization of a compact tertiary structure, and putative HS–glycanation sites that occur carboxyl terminally, close to the anchor structure that links the protein to the cell surface (David, 1993). The precise functions of the various syndecan- and glypican-like core proteins remain unknown, but it is generally assumed that, at a minimum, they serve to target functionally critical HS chains to their positions on the cell surface and regulate their expressions at these sites.

Studies on the polarized expression of membrane proteins in epithelial cells have revealed that in many instances the transport of proteins to specific membrane compartments involves defined sorting signals (reviewed by Bomsel and Mostov, 1991). The best-characterized sorting signal so far is the GPI anchor, which directs proteins to the apical surface of several epithelial cell types. In other cases cytoplasmic domains have been shown to be responsible for basolateral deliveries, and recent studies, demonstrating that cytoplasmic tail deletions affect the transport and basolateral targeting of syndecan-1 in transfectant MDCK cells (Miettinen et al., 1994), suggest that this may be the case for the syndecans. In such a scheme particular proteoglycan forms might be specialized for particular functions, not because of the unique binding and catalytic properties of their HS chains, but because of their specific locations at a functionally relevant place on the cell surface, i.e., glypicans in apical and syndecans in basolateral compartments. Previous studies have shown that all syndecans and glypican isolated from rat and human vascular endothelial cells can be substituted with heparan sulfate chains that bind to antithrombin III (Kojima et al., 1992; Mertens et al., 1992), suggesting that several different core proteins isolated from one cell type may carry structurally and functionally similar HS chains. Yet, HSPGs on the luminal surface of the endothelium would be the first candidates to activate circulating antithrombin III.

We therefore initiated a study of the subcellular localization of the different cell surface–associated HSPGs, and more specifically of glypican, in CaCo-2 and transfectant MDCK cells, polarized cell types that have often been used as models for the study of protein trafficking and sorting signals. The results of vectorial labeling experiments indicate that the syndecans expressed by CaCo-2 cells are restricted to the basolateral membrane compartment of these cells. Glypican, in contrast, is expressed in the apical membrane and, unexpectedly, also in the basolateral compartment, endogenously in CaCo-2 cells and after transfection in MDCK cells. Site-directed mutagenesis of the glypican cDNA used for the transfection studies and analysis of the subcellular expression of glypican forms that are substituted with one, two or three HS chains and of non glycanated forms indicates that substitution of the glypican core protein with HS interferes with the apical sorting signal that is provided by the GPI anchor of this protein. The unexpected differential subcellular expressions of the proteoglycan and nonglycanated forms of glypican imply that modulated substitution with HS provides a potential means of controlling the subcellular expression and functional availability of this membrane protein.

Materials and Methods

Culture and Metabolic Labeling of the Cells

Human colon carcinoma cells (CaCo-2) (Zwiebaum, A., INSERM Villejuif, France), wild type strain II MDCK (Simons, K., EMBL, Heidelberg, Germany), and transfectants derived from these MDCK cells were grown in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum. For biotinylation experiments, the cells were plated in 24.5 mm filter chambers (Costar Corp., Cambridge, MA) provided with collagen-treated nitrocellulose (Transwell-Col, 0.4 μm pore) or tissue culture-treated polyester membranes (Transwell-Clear, 0.4 μm pore), at a density of 200,000 cells/well. The medium was renewed every other day and experiments were performed 8 d after plating. The apical expression of carcino embryonic antigen (CEA) in CaCo-2 cells (see Results) and of gp 80 (Mostov et al., 1992) in MDCK cells (not shown) indicated that the cells were well polarized at the initiation of the experiment. For the chlorate-treatment of the cells, the standard DME medium was replaced by a low sulfate-DME (0.08 mM SO_4^{2-}) containing 50 mM chlorate on day two after plating, and this medium was renewed every other day. On the 8th day, the confluent monolayers were labeled for 24 h with $^{35}SO_4^{2-}$ (50 μCi/ml; New England Nuclear, Boston, MA) in low sulfate-medium (0.08 mM SO_4^{2-}), with both $^{35}SO_4^{2-}$ (50 μCi/ml) and ^{3}H-glucosamine (20 μCi/ml) in low sulfate-medium (chlorate-treated cells), or with [^{35}S]methionine and [^{35}S]cysteine (25 μCi/ml each; New England Nuclear) in methionine and cysteine-free RPMI 1640 medium (ICN Biomedicals, Costa Mesa, CA). For the time course label and chase experiments, the CaCo-2 cells were labeled with $^{35}SO_4^{2-}$ (200 μCi/ml) in low-sulfate medium for various time periods up to 4 h, or labeled for 24 h, and then chased in standard culture medium without precursor for various time periods up to 24 h.

Monoclonal Antibodies

The mAb used for the immunopurification and detection of the proteoglycans were all raised against proteoglycans of human origin: mAb 3G10 (anti-Δ-HS), mAbs S1 and 1G11 (glypican), mAb 2E9 (syndecan-1 and -3), mAbs 6G12 and 10H4 (fibroglycan/syndecan-2), mAb 1C7 (syndecan-3), mAb 8G3 (amphiglycan/syndecan-4), and all have been described before (De Boeck et al., 1987; Lories et al., 1989, 1992; David et al., 1990, 1992). The anti-human carcinoembryonic antigen mouse monoclonal antibody (DAKO-CEA, A5B7) was obtained from DAKO (Glostrup, Denmark).

Proteoglycan Extraction and Characterization

Triton X-100 (TX-100) extraction buffer contained 10 mg/ml TX-100, 150 mM NaCl, 10 mM Tris/HCl, pH 8.0, 50 mM 6-aminohexanoic acid, 5 mM *N*-ethylmaleimide, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml of pepstatin A, pH 7.4. The octylglucoside (OG) extraction buffer had the same composition except that TX-100 was replaced by 60 mM octylglucoside (Boehringer, Mannheim, Germany). Ion-exchange, gel-filtration, liposome-incorporation, immunoprecipitation and -blotting procedures used to isolate and characterize the proteoglycans from cell extracts or media fractions were as described before (Lories et al., 1989; Mertens et al., 1992).

Biotinylation and Detection of Biotinylated Proteoglycan

Filter-grown monolayers were assayed for impermeability by adding [^{3}H]inulin (Amersham, Buckinghamshire, England) to the apical compartment of the filter chamber (Sargiacomo et al., 1989). Metabolic labeling was initiated in monolayers showing a permeability less than 1%, and 24 h later the cultures were used for vectorial biotinylation experiments, as described by Sargiacomo et al. (1989). Filters were placed on ice, rinsed three times with cold phosphate-buffered saline containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS-CM), and a freshly made solution of sulfo-NHS-

biotin (0.5 mg/ml in PBS-CM) (Pierce, Rockford, IL) was added to the apical or basolateral chamber. The cells were incubated for 20 min at 4°C, whereupon the biotinylation procedure was repeated once. The reaction was quenched by removing the biotin solution and adding serum-free culture medium (10 min), followed by three washes of the cells with PBS-CM, and finally by extraction of the cells with TX-100 or OG buffer (2× for 20 min). The detergent extracts were cleared by centrifugation (10,000 *g*; 10 min) and analyzed for their total and biotinylated proteoglycan contents.

In one procedure all proteoglycans were bulk purified from the detergent extracts by ion-exchange chromatography on Mono-Q, treated with heparitinase and chondroitinase ABC, separated on 6–20% SDS–polyacrylamide gradient gels, and electrotransferred to a Zeta-probe membrane. After overnight inactivation in 1% Tween-20, 0.5% casein, 600 mM NaCl, 10 mM Na_2HPO_4, pH 7.4, the membrane was incubated with alkaline phosphatase-linked avidin (Tropix, Bedford, MA) (1/5,000) in PBS supplemented with 0.5% casein and 1% Tween-20. The membrane was rinsed, incubated with the chemiluminescent substrate AMPPD (Tropix) and biotinylated core protein was detected by exposure to Hyperfilm-ECL (Amersham, Belgium, Gent) as described previously (Mertens et al., 1992).

In the other procedure, each proteoglycan was individually purified from the detergent extracts by immunoabsorption on specific antibody. In some experiments the proteoglycans were pre-purified by adsorption on DEAE-Trisacryl M, washing with 0.1% TX-100, 0.2 M NaCl, 50 mM Tris/HCl, pH 7.4, and elution in 0.1% TX-100, 1 M NaCl, 50 mM Tris/HCl, pH 7.4. Before immunopurification the samples were adjusted to 0.15 M NaCl and supplemented with BSA (50 μg/ml), heparin (10 μg/ml), and chondroitin sulfate (10 μg/ml). Immunopurification of the proteoglycans on immobilized monoclonal antibodies was performed as described before (Lories et al., 1989). Immunopurified proteoglycan was dissolved in PBS supplemented with BSA (50 μg/ml) and 0.1% TX-100, and mixed with immobilized streptavidin (Pierce Chemical Co., Rockford, IL). After incubation for 3 h at 4°C, the streptavidin beads were washed three times with PBS containing BSA and 0.1%TX-100. Streptavidin-bound and nonbound materials were analyzed for their radiolabel-content by liquid scintillation counting. Alternatively, the streptavidin-bound label was solubilized by boiling in 20 mM Tris/HCl buffer, pH 7.4, containing 2% SDS, 10% glycerol, and 0.05% bromophenol blue. These samples were analyzed by SDS-PAGE in a 10% homogeneous acrylamide gel, and autoradiography.

Liposome Incorporation and PI-Phospholipase C Digestion

Immunopurified $^{35}SO_{32}$-labeled proteoglycans were incorporated into lipid vesicles as described before (Lories et al., 1987). Liposome-incorporated proteoglycans were separated from the nonincorporated proteoglycans on a sucrose gradient (Andres et al., 1989). Liposome-incorporated fractions in 50 mM Tris/HCl, pH 7.5, were supplemented with 50 mM octylglucoside, 10 mM EDTA and proteinase inhibitors (see above) and treated with 50 mU of phosphatidyl inositol-specific phospholipase C (Boehringer, Mannheim, Germany) for 3 h at 37°C. After the digestion, treated and control samples were retested for liposome incorporation as described above.

Transfection of Glypican into MDCK Cells

The 64K3 cDNA coding for wild type human glypican (David et al., 1990) and derivative mutant cDNAs (see below) were integrated in the XbaI-HindIII restriction sites of the pRcRSV eukaryotic expression vector (Invitrogen, Leek, The Netherlands). The constructs and control empty pRcRSV vectors were linearized with ScaI before transfection. MDCK cells were washed three times with PBS, and 3 $\times 10^6$ cells were electroporated (capacity: 960 μF, voltage: 260 V) with 30 μg of plasmid DNA. Cells were cultured in normal culture medium supplemented with penicillin (100 U/ml; GIBCO BRL) and streptomycin (100 μg/ml; GIBCO BRL) for 24 h before selection in medium supplemented with G418 (0.480 mg/ml; Boehringer). The G418-resistant cell populations were tested for glypican expression in dotblot and Western blot assays and by immunocytochemistry using the mAbs S1 and 1G11.

Construction of the Glypican Mutants

Sequences coding for the three potential HS attachment sites near the carboxyl terminus of glypican were mutated. The bases 1,677 to 1,695 of the 64K3 cDNA, cloned in Bluescript, were changed from AGC GGC TCG GGC AGC GGT to AGC GGC TCG GGC ACC GGT, to AGC GGC ACG GGC ACC GGT, or to ACC GGC ACG GGC ACC GGT, using the Transformer site-directed mutagenesis kit (Clonetech, Palo Alto, CA). These mutations, which replace the triple SG repeat in the protein by the sequences SGSGTG, SGTGTG or TGTGTG, were confirmed by restriction analysis and DNA-sequencing. The mutated 64K3cDNAs were ligated into the pRcRSV vector and used for the transfection of MDCK cells as described above.

A syndecan-4/glypican chimera (35KN/64KC) was constructed by PCR, starting from parts of the cDNAs for these proteins, subcloned in pBluescript, and using the primers T7 and TGC CAG GAG CTC CGT TCT CT (syndecan-4) and the primers T3 and CCC AGA GCT CTC AGA GCA GG 5 (glypican). The PCR products were verified by sequencing, and ligated in pRcRSV using the enzymes HindIII, SacI, and XbaI. The resulting chimeric cDNA insert includes residues 1–458 of the 35K17 sequence (David et al., 1992), the GAGCTC sequence (SacI), and residues 1785–2443 of the 64K3 sequence (David et al., 1990). Translation of this construct is predicted to initiate at the start ATG codon, preceded by the Kozak sequence, of the syndecan-4 cDNA and to be terminated by the TAA stop codon of the glypican cDNA, to yield a chimera composed of residues 1–145 (the signal peptide and ectodomain) of the predicted sequence for human syndecan 4 and of residues 521–558 (C-terminus) of the predicted sequence for human glypican. All potential heparan sulfate glycanation sites in this construct are derived from syndecan-4.

Results

Integral Membrane Proteoglycans Expressed by CaCo-2 Cells

Confluent monolayers of CaCo-2 cells express several different forms of cell surface heparan sulfate proteoglycan. This is illustrated by Western blot analyses of the liposome-intercalatable proteoglycan fraction from these cells, using various syndecan, glypican and Δ-HS–specific monoclonal antibodies (Fig. 1). Staining of the heparitinase-treated fractions with mAb 3G10, an antibody that recognizes the desaturated uronates on the HS "stubs" that remain associated with the core proteins after a heparitinase digestion, revealed multiple prominent bands with apparent molecu-

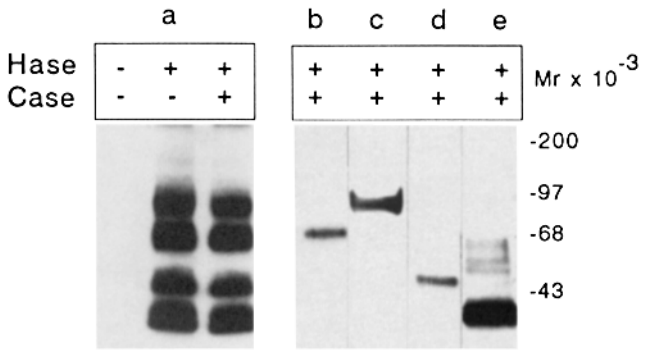

Figure 1. Identification of the cell surface HSPGs in CaCo-2 cells. Hydrophobic proteoglycans, isolated from Caco-2 cells were treated (+) or not treated (−) with heparitinase (*Hase*) and/or chondroitinase ABC (*Case*). Samples were analyzed by electrophoresis in SDS–polyacrylamide gradient gels, followed by electrotransfer to a Zeta-probe membrane and immunostaining with mAbs raised against the cell surface–associated proteoglycans from human lung fibroblasts: (*a*) mAb 3G10, an anti-ΔHS antibody that reacts with any HSPG core after treatment with heparitinase; (*b*) mAb S1 for glypican; (*c*) mAb 2E9 for syndecan-1 and -3; (*d*) mAb 6G12 for syndecan-2; (*e*) mAb 8G3 for syndecan-4.

lar masses of ~100, 64, 46, and 35 kD. The 100, 64, 46, and 35-kD bands were identified as, respectively, syndecan-1, glypican, syndecan-2 (fibroglycan) and syndecan-4 (amphiglycan/ryudocan) by various core protein–specific antibodies. During culture, CaCo-2 cells also release HSPGs into the culture media (not shown). The single major ~60-kD core protein stained by mAb 3G10 in Western blots of heparitinase digests of these fractions was identified as glypican, suggesting that, as in many other cell types, this proteoglycan is shed from the surface of CaCo-2 cells.

Polarity of the Expression of the Membrane-associated HSPGs in CaCo-2 Cells

The distribution of the major membrane-associated heparan sulfate proteoglycans over the apical and the basolateral cell surface domains of these epithelial cells was investigated by growing the cells on semi-permeable filters in a Transwell Costar culture system, and submitting the cells to domain-specific biotinylation. The biotinylated cell surface proteoglycans were analyzed as described in Materials and Methods, and the results of these experiments are illustrated in Fig. 2 and summarized in Table I.

Fig. 2 shows the Western blots of proteoglycan fractions isolated from apically or basolaterally biotinylated cells, developed by chemoluminescent staining using avidin-linked alkaline phosphatase. Basolateral biotinylation yielded multiple strongly labeled core proteins, apical biotinylation mostly a core protein of ~64 kD, suggesting that glypican was the main proteoglycan expressed at the apical membrane surface. For three major cell surface proteoglycans that were immunopurified from CaCo-2 cells, Table I lists the fraction of the total radiolabel that was retained on immobilized streptavidin when this proteoglycan was isolated from metabolically labeled cells that were biotinylated from either the apical surface, the basolateral surface, or not biotinylated. The results of several independent experiments indicate that biotinylated forms of $^{35}SO_3^{2-}$-labeled syndecan-1 and -4 were virtually restricted to the basolateral membrane compartment of the CaCo-2 cells. The $^{35}SO_3^{2-}$-labeled glypican, in contrast, was readily detectable in the apical compartment, but also in the basolateral compartment. Actually, three to four times more glypican was biotinylated in the basolateral than in the apical labeling procedure (A/B = 0.274; SEM = 0.053; n = 6).

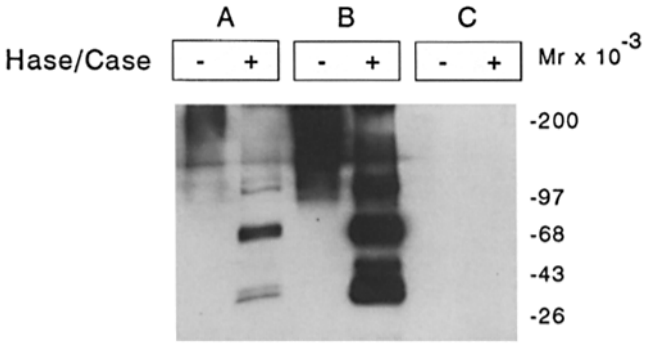

Figure 2. Characterization of the core proteins of the domain-specific biotinylated proteoglycans expressed in CaCo 2 cells. Cells cultured in Transwell Costar culture plates and labeled with $^{35}SO_4^{2-}$ for 22 h, were biotinylated from either the apical (*A*) or basolateral (*B*) compartment; controls (*C*) were not biotinylated. Cells were lysed with TX-100 buffer and the proteoglycans were purified from these extracts by ion-exchange chromatography on Mono Q. The ^{35}S-containing fractions were pooled, concentrated and treated with heparitinase (*Hase*) and chondroitinase ABC (*Case*). Treated (+) and nontreated (−) samples were analyzed by electrophoresis in a gradient SDS–polyacrylamide gel, followed by transfer to a Zeta-probe membrane. The biotinylated proteoglycans were detected by staining with an avidin-linked alkaline phosphatase and by chemiluminescence.

Table I. Polarity of the Expression of the HSPGs in CaCo 2 Cells

Cell Surface HSPG Expression in CaCo-2 Cells Biotinylated Fraction (Percent of total proteoglycan)						
Form	Detergent	A	B	C	A-C/B-C	*n*
Glypican	TX	13	50	1	0.241	3
	OG	17	59	1	0.275	6
Syndecan-1	TX	1	16	0	0.062	2
	OG	2	23	1	0.045	1
Syndecan-4	TX	2	18	1	0.058	1
	OG	3	23	1	0.090	1

Cells were cultured in Transwell Costar culture plates and labeled with $^{35}SO_4^{2-}$ for 22 h. Each separate experiment involved three cultures, analyzed in parallel. Test cells were biotinylated from either the apical (*A*) or basolateral (*B*) compartment. Controls were not biotinylated (*C*). Cells were lysed with TX-100 buffer (*TX*) or OG buffer (*OG*), and the proteoglycans were immunopurified from these extracts. Biotinylated and non-biotinylated proteoglycans were separated on immobilized streptavidin and analyzed for their ^{35}S-content. The radioactivity associated with the biotinylated fraction (streptavidin-bound label) was expressed as a percentage of the total label recovered for that particular proteoglycan from the antibody column. Lack of 100% recovery was also observed when the biotinylations were performed in the presence of detergent, and reflects the relative inefficiency of the biotinylation procedure rather than the presence of substantial intracellular pools (not shown). The number of separate experiments from where these numbers (or averages) were obtained is indicated (*n*).

To investigate the polarity of the shedding, glypican was also immunopurified from the apical and basolateral culture media after 24 hours of labeling with $^{35}SO_4^{2-}$. The ratio of apically shed glypican to basolaterally shed glypican was found to be ~0.38 (SEM = 0.046; n = 4) (Table II), indicating that glypican was shed to both culture compartments, but in relative amounts that were slightly different from the distribution of this proteoglycan over the corresponding membrane domains, with more glypican released to the apical medium than expected from the membrane distribution. All together, ~70% of the total amount of $^{35}SO_3^{2-}$-glypican synthesized during the 24 h labeling period had ended up in the basolateral compartment of the culture system (basolateral membrane + basolateral culture medium).

The polarity of the expression of the syndecans and glypican in CaCo-2 cells was not influenced by the collagen coats used in the above experiments, as experiments with cells cultured on noncoated filters yielded similar results. Similar results were also obtained whether the cells were lysed in the filter chamber or lysed after excision of the filter, excluding the possible contribution of cells growing on the vessel walls (Zurzolo, 1993). Finally, the polarity of the heparan sulfate proteoglycan expression was also found to be similar in cells cultured for 8 or for 14 d (results not shown).

Unconventional Sorting of Glypican

Since most, if not all, GPI-linked proteins in epithelial cells are restricted to the apical surface (Le Bivic et al.,

Table II. Polarity of the Shedding of Glypican in CaCo-2 and Glypican-transfected MDCK Cells

Source	Type	Label	A/B	*n*
		Polarity of the Shedding of Glypican Ratios of the apical and basal shed glypican		
CaCo-2	endogenous	sulfate	0.380	4
		methionine	0.564	2
		glucosamine	0.237	2
MDCK	wild	sulfate	1.010	3
		methionine	0.935	2
	two-chain	sulfate	2.160	3
	one-chain	sulfate	2.980	4
	no-chain	methionine	9.046	3
	chimera	sulfate	0.576	3

Glypican was immunopurified from the apical and basolateral culture media of cells that were cultured on Transwell Costar plates and labeled with $^{35}SO_4^{2-}$, ^{3}H-glucosamine, or [^{35}S]methionine and cysteine. For the experiments with CaCo-2 cells glypican was pre-purified from the media by absorption on DEAE. For the experiments with MDCK cells glypican was immunopurified directly from the media. Similar progressive increases in A/B ratios with decreasing chain numbers were also observed when glypican from proteoglycan-transfectant MDCK cells was pre-purified on DEAE (not shown). The chainless form of glypican, however, failed to bind to DEAE. The numbers represent averages of the ratios of the amounts of label recovered from the apical and basolateral media. The number of independent experiments from where these averages were derived is indicated (*n*).

1989; Mostov et al., 1992), the mainly basolateral distribution of glypican was unexpected. To confirm that the basolateral glypican was GPI linked, glypican isolated from basolaterally biotinylated cells was incorporated into liposomes and tested for susceptibility to phosphatidylinositol-specific phospholipase C (Fig. 3). After recentrifugation of the liposomes on sucrose gradients, biotinylated glypican was recovered from the bottom fractions when treated with PLC, but from the top fractions when left untreated. Biotinylated syndecan-1, isolated from the same cells, was recovered from the top fractions and not affected by the PLC treatment.

On the other hand, GPI-linked proteins are not efficiently solubilized by certain detergents, including cold solutions of TX-100, possibly because their lipid anchors associate with membrane domains of unique lipid compositions (Hooper and Turner, 1988; Brown and Rose, 1992). CaCo-2 cells cultured on Transwells were therefore biotinylated and successively extracted with cold TX-100 and octylglucoside. Dot blot analyses and quantitative immunopurifications of the HSPGs from these extracts revealed that glypican was significantly, but only partially, solubilized in cold TX-100 (~33%), while syndecan-1, which is a transmembrane proteoglycan, was quantitatively extracted by TX-100 (>95%). Analysis of the biotinylated fractions in the different extracts showed that neither the glypican extracted with TX-100 (Table I) nor the residual glypican extracted with octylglucoside (A/B = 0.171) were apically polarized. Yet, similar biotinylation and successive extraction experiments with cold TX-100 and octylglucoside indicated that, as predicted for glypiated proteins, recovery of biotinylated human carcinoembryonic antigen (CEA) was virtually limited to apical labeling reactions and to the octylglucoside extracts of these CaCo-2 cells (Fig. 4).

Biotin-accessible $^{35}SO_3^{2-}$-glypican could also be recovered from both the basolateral and apical surfaces of the CaCo-2 cells after brief labeling periods (0.5–4 h), whereby the accumulation of this proteoglycan on the basolateral

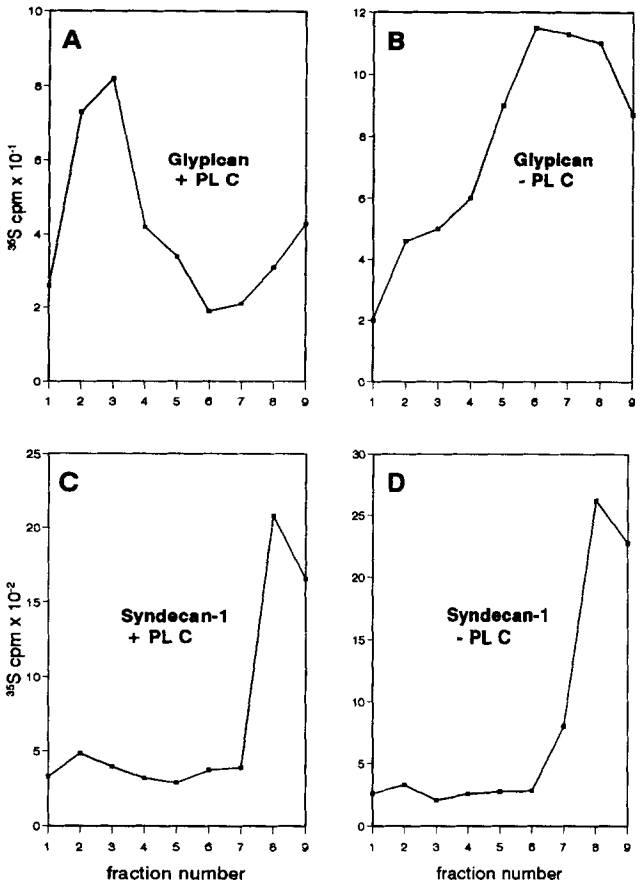

Figure 3. Sensitivity to phospholipase C of the basolaterally expressed glypican and syndecan 1. CaCo-2 cells cultured on Transwell Costar culture plates and labeled with $^{35}SO_4^{2-}$ for 22 h, were biotinylated from the basolateral compartment. Glypican (*A* and *B*) and syndecan-1 (*C* and *D*) were immunopurified from the cell extracts on antibody columns and incorporated into liposomes. Incorporated materials were separated from the nonincorporated materials by centrifugation on a sucrose gradient, before treatment with phospholipase C (PLC). All label comigrated with the liposomes (not shown). The liposome-incorporated fractions were treated (*+PLC*) or nontreated (*−PLC*) with PLC, treated (*A* and *C*) and nontreated samples (*B* and *D*) were recentrifugated on a sucrose gradient, and each fraction of the gradient was analyzed for its streptavidin-binding ^{35}S-content.

cell surface exceeded that on the apical surface from the initial measurements on (Fig. 5 *A*). Pulse–chase experiments, whereby metabolically labeled cultures were biotinylated after various time periods of culture in precursor-free medium, indicated that apical and basolateral biotin-accessible $^{35}SO_3^{2-}$-glypican forms were cleared from the cell surface at similar rates (Fig. 5 *B*). Virtually all (90%), of the metabolically labeled glypican that was cleared from the cells during the chase period was recovered in the culture media. Most of this shed glypican was accumulating in the basolateral medium (ratio of apical over basolateral shed $^{35}SO_3^{2-}$-glypican = 0.472). Other pulse–chase experiments, in which the cells were vectorially biotinylated at the end of the metabolic labeling pulse and then chased, indicated that the apically or basolaterally biotinylated glypican was mostly released to the ipsilateral medium compartment (in both cases ~75% of the total shed biotinylated proteogly-

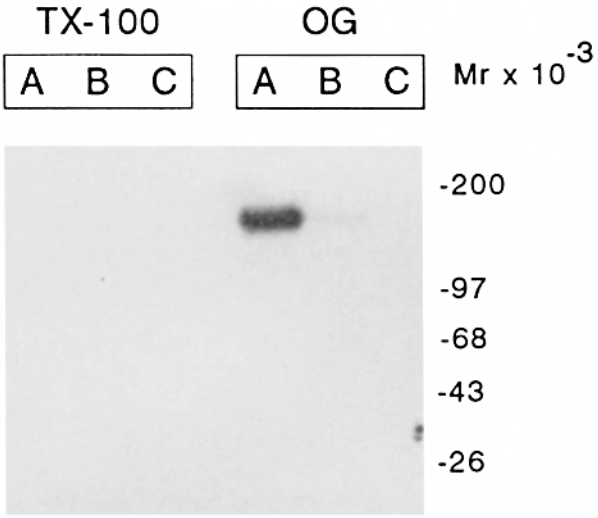

Figure 4. Detergent solubility and polarity of the expression of CEA in CaCo-2 cells. Caco-2 cells grown on Transwell Costar culture plates were biotinylated from either the apical (*A*) or basolateral (*B*) compartment; controls (*C*) were not biotinylated. Cells were extracted successively with cold TX-100 (TX-100) and octylglucoside (*OG*). All biotinylated components present in both the TX-100 and OG extracts were concentrated on immobilized streptavidin. After several washes, the streptavidin beads were boiled for 10 min in 2% SDS–sample buffer and analyzed by electrophoresis on a 4% Nusieve agarose gel. The samples were transferred to a Zeta-probe membrane and immunostained with an anti-CEA mAb.

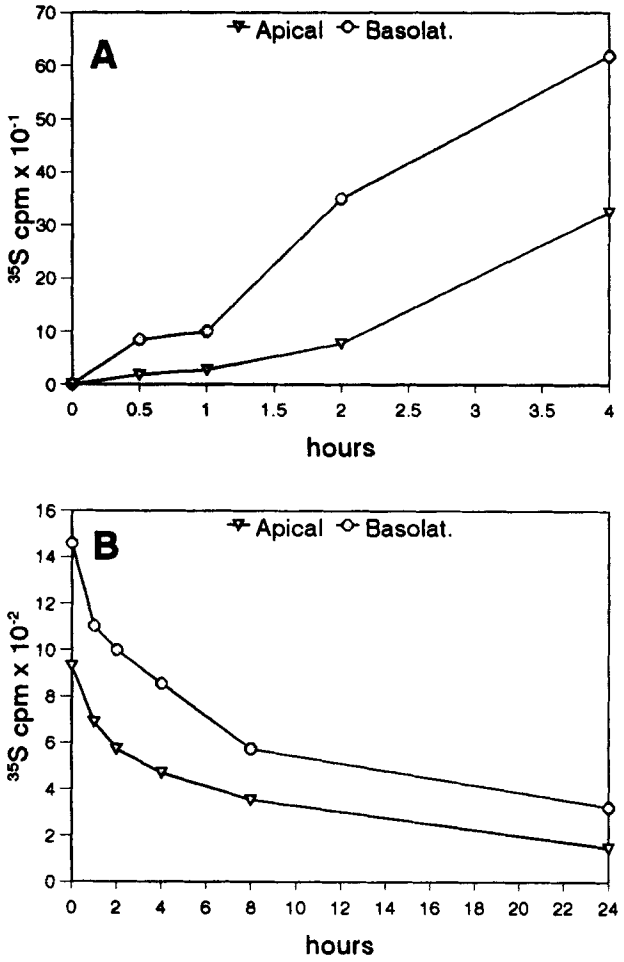

Figure 5. Kinetics of the labeling and chase of label from the biotinylated apical and basolateral glypican fractions in CaCo-2 cells. CaCo-2 cells were grown on Costar Transwell culture plates and biotinylated from either the apical or the basolateral compartment after varying times of $^{35}SO_4$ incorporation (*A*), or after varying times of chase of the label from cells labeled for 24 h (*B*). Control cells were not biotinylated. The biotinylated fractions were isolated and analyzed as described in Table I.

can). Shedding to the contralateral compartment (~25% of the total shed biotinylated proteoglycan) in these experiments could be attributed to a transcytotic pathway, since in control experiments no diffusion to the contralateral medium compartment could be measured when labeled glypican was added to the apical or basal culture medium and label accumulating in the contralateral medium was measured for up to 24 h. Moreover, from this data we calculated that the relative sizes of the apical and basolateral biotinylated membrane-associated pools (A/B = ~0.25) and the combined magnitudes of the ipsilateral and contralateral sheddings could account for higher apical/basal glypican ratios in media compartments than in cell compartments [predicted ratio for the media = (0.25 × 0.75) + (1 × 0.25)/(1 × 0.75) + (0.25 × 0.25) = 0.538]. These data suggested that sorting rather than differential stabilization or turnover mechanisms were at the basis of this unexpected bipolar expression of glypican.

Finally, transfection experiments, in which a cDNA coding for human glypican was introduced into polarized MDCK cells, indicated that the basolateral expression of glypican was not a particularity of the CaCo-2 cell system. Western blotting demonstrated that the glypican expressed by the transfectant clones had a core protein of 64 kD, that it was sensitive to heparitinase only, and that it was shed into the culture medium (Fig. 6). A domain-selective biotinylation of two different MDCK clones showed that also in this cell system the $^{35}SO_3^{2-}$-glypican was distributed over both the apical and basolateral surfaces (Table III). Quantification of the amounts of $^{35}SO_3^{2-}$-glypican accumulated into the culture media of these cells revealed that, as in CaCo-2 cells, this proteoglycan was shed to both the apical and the basolateral culture media (Table II).

These experiments confirmed that in polarized cells glypican did not behave like most other GPI-linked proteins.

The HS Chains Interfere with the Apical Sorting of Glypican

Current models for the apical sorting of GPI-anchored proteins (Simons and Wandinger-Ness, 1990; Brown and Rose, 1992; Hooper, 1992) propose that GPI-anchored proteins and glycolipids associate in clusters during transport through the Golgi, whereby the GPI-linked molecules would become insoluble in TX-100 at the time the clusters are formed. These clusters would then segregate, associate with the elements of a specialized sorting machinery and be directed to the apical surface of polarized epithelial cells. Glypican does not fit in this model, escaping in part from clustering into these domains (partial Triton solubil-

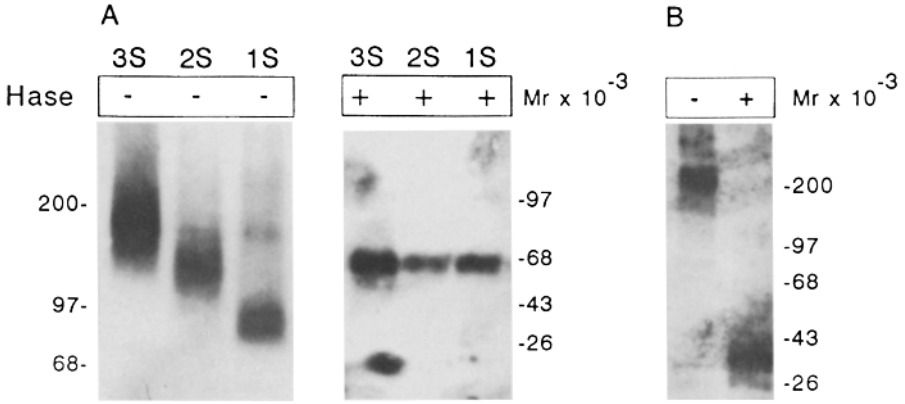

Figure 6. Characterization of wild type and mutant glypicans expressed in transfectant MDCK cells. MDCK cells transfected with the wild type (*3S*), the SGSGTG (*2S*) or the SGTGTG (*1S*) mutant form of the 64K3 cDNA for human glypican (*A*), or the chimera (*B*) were cultured on plastic substrates and solubilized with OG buffer. Proteoglycan isolated from the detergent extract (by absorption on DEAE) was not treated (−) or treated (+) with heparitinase (*Hase*), fractionated by SDS-agarose (glypican), or polyacrylamide (chimera) gel electrophoresis, and transferred to a Zeta-probe membrane. The transfectant proteins were visualized by immunostaining using the mAbs S1 and 1G11 for glypican (*A*) or 8G3 (syndecan-4) for the chimera (*B*). Nontransfectant MDCK cells do not express the S1, 1G11, and 8G3 epitopes (not shown).

ity of the cell surface–exposed forms) and failing to segregate (apical and basolateral expressions). Glypican may, for example, associate with a heparin-binding protein that contains basolateral determinants and mediates its transfer to the basolateral membrane. Alternatively, HS chains with attachment sites located near the carboxyl terminus of the core protein, close to the membrane, might prevent the interaction of glypican with the sorting components. To test these hypotheses, cells were treated with chlorate, to inhibit glycosaminoglycan sulfation, and glypican mutants of differing HS acceptor site valencies were constructed and expressed in MDCK cells.

Chlorate treatment of the CaCo-2 cells reduced the sulfation of the HSPGs to 6% of the normal level (not shown), but did not affect the polarized expression of glypican (nor that of syndecan-1) in these cells. In chlorate-treated cells, ^{3}H-glucosamine–labeled glypican was found at the basolateral surfaces (Table IV) and was shed in both the apical and basolateral compartments (not shown), in similar proportions as in control cells, suggesting that normally sulfated chains were not required for interacting with a hypothetical basolateral sorting machinery. The ratios that were obtained, however, were slightly different from those obtained in ^{35}S-sulfate–labeling experiments. Cellular ^{3}H-glypican was even more preponderant over the basolateral surface (A/B ~ 0.170) than the ^{3}S-glypican (A/B = 0.274). This suggests some differences in the sulfation of glypican between different culture compartments, but without relationship to sorting.

To test whether the HS chains might prevent the polarized expression of glypican, the three serines at the potential HS attachment sites near the carboxyl terminus were converted into threonines, to create two-chain, one-chain, and unsubstituted forms of glypican. Autoradiographic analyses (not shown) and Western blot analyses of wild type and mutant glypican forms, immunoprecipitated from transfectant MDCK cells, indicated a progressive increase in average relative molecular weight consistent with the predicted increase in chain valency (Fig. 6). The one-, two-, and three-chain forms of glypican had core proteins of identical sizes, and heparan sulfate was the only glycosaminoglycan identifiable in these proteoglycans. The sizes of the free heparan sulfate chains, prepared by alkaliborohydride of the proteoglycans, were similar for the three forms, whereas the sizes of the chain clusters, prepared by trypsin digestion, increased with the predicted in-

Table III. Polarity of the Cell Surface Expression of Wild Type and Mutant Glypican in MDCK Transfectants

Cell Surface Expression of Glypican in Transfectant MDCK Cells
Ratios of the apical and basolateral biotinylated fractions

Form	Label	A-C/B-C	*n*	$HS_a + HS_b = HS_t$
Wild type	sulfate	0.565	(5)	108 + 192 = 300
Two-chain	sulfate	0.970	(7)	98 + 102 = 200
One-chain	sulfate	1.180	(4)	54 + 46 = 100
No chain	methionine	1.650	(2)	
Chimera	sulfate	0.543	(3)	

Cells were cultured in Transwell Costar culture plates and labeled with $^{35}SO_4^{2-}$ or with [^{35}S]methionine and cysteine for 22 h. Test cultures were biotinylated from either the apical or basolateral compartment. Control cultures were not biotinylated. The cells were lysed with OG buffer, and glypican was directly immunopurified from these extracts. The apical (*A*) and basolateral (*B*) biotinylated fractions were isolated by incubation with immobilized streptavidin and analyzed for their ^{35}S-content. The amount of label retained on streptavidin in isolates from control cultures (*C*) was substracted from these numbers. The number of separate experiments from where these average ratios were obtained has been indicated (*n*). In the last column fictive numbers for the apical (HS_a), basolateral (HS_b) and total (HS_t) HS values are given that correspond to the A/B ratios that were experimentally obtained for the corresponding proteoglycan forms, and to a model whereby the cells express constant amounts (i.e., 100 copies) of glypican coreprotein substituted with either three, two or one structurally similar HS chains.

Table IV. Effect of Chlorate on the Polarity of the Expression of Glypican and Syndecan-1 in CaCo-2 Cells

Effect of Chlorate on the Cell Surface Expression
of the HSPG in CaCo-2 Cells
Biotinylated Fraction (Percent of total proteoglycan)

Forms	Treatment	A	B	C	A-C/B-C
Glypican	none	3.3	20.2	0.5	0.163
	chlorate	3.0	17.3	0.4	0.173
Syndecan-1	none	1.2	15.9	0.1	0.069
	chlorate	2.1	11.0	0.3	0.190

Cells were cultured in Transwell Costar culture plates, treated or not treated with chlorate, and labeled with ^{3}H-glucosamine for 22 h. Test cells were biotinylated from either the apical (*A*) or basolateral (*B*) compartment. Controls were not biotinylated (*C*). The cells were lysed with octylglucoside buffer and the proteoglycans were immunopurified from these extracts. Biotinylated and nonbiotinylated proteoglycans were separated on immobilized streptavidin and analyzed for their ^{3}H-content. The radioactivity associated with the biotinylated fraction (streptavidin-bound label) is expressed as a percentage of the total label recovered for that particular proteoglycan from the antibody column.

crease in maximal chain number in these clusters (not shown). The TGTGTG mutant had an apparent molecular weight of ~64 kD (Fig. 7), and was not substituted with glycosaminoglycan as revealed by its resistance to heparitinase and chondroitinase ABC (not shown). These glypican mutants were then tested for the polarity of their expression in ^{35}S-sulfate and in [^{35}S]cysteine plus [^{35}S]methionine-labeled cells. The chainless mutant was mostly detected at the apical surface of the transfectant cells (Fig. 7 and Table III), and the shedding of this mutant was nearly limited to the apical compartment (Fig. 7 and Table II). Taken together, the majority (~90%) of the chainless glypican mutant was sorted to the apical (membrane and culture medium) compartment. The one- and two-chain mutant proteoglycan forms of glypican, in contrast, had distributions that were intermediate between that of the wild type proteoglycan and that of the chain-less form (Tables II and III). The results of these experiments suggest that the presence of the HS chains interferes with the apical sorting signal provided by the GPI anchor and prevents the polarized expression of glypican in MDCK cells.

As a further test of this interpretation, we constructed an artificial glypiated HSPG in the form of a syndecan-4/glypican chimera. This construct was composed of the complete ectodomain of this syndecan, providing the sites for substitution with HS, and the 38 last residues of glypican, providing the determinants that are required for the GPI anchor attachment (the site for the transamidation reaction and the hydrophobic signal peptide-like COOH-terminal sequence). Syndecan-4 was chosen to make this construct because the sites for substitution with HS in this syndecan are more proximal to the cell surface than in the other forms, possibly providing for a better imitation of the display of the chains in glypican. After transfection into MDCK cells, three independent cell clones expressed the chimera in a bipolar fashion, both on the cell surface and in the culture media (Tables II and III), consistent with the proposal that bipolar expression of glypiated proteoglycans is primarily due to the presence of the heparan sulfate chains.

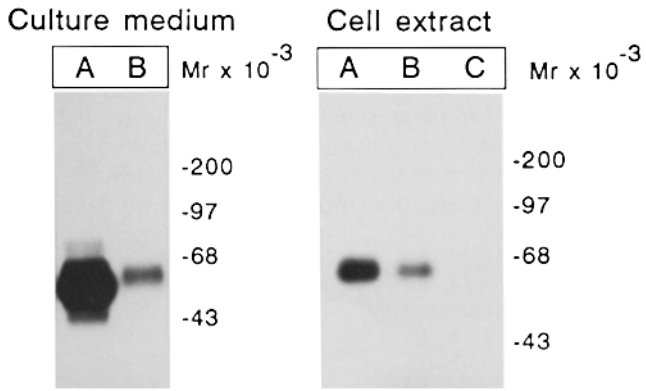

Figure 7. Polarity of the expression of the chainless–glypican mutant in transfectant MDCK cells. The MDCK clone expressing the TGTGTG-glypican mutant was grown on Transwell Costar culture plates and labeled with [^{35}S]cysteine and [^{35}S]methionine for 22 h. Apical (*A*) and basolateral (*B*) culture media were collected separately. The cells were biotinylated from either the apical (*A*) or basolateral (*B*) compartment; controls (*C*) were not biotinylated. Cells were lysed with OG buffer. Glypican was immunopurified directly from the culture media and cell extracts. The biotinylated immunopurified cellular glypican was isolated on immobilized streptavidin. Total apical and basolateral medium glypican, and apically and basolaterally biotinylated cellular glypican were analyzed by SDS-PAGE and autoradiography without any prior heparitinase digestion.

Discussion

This study investigated the expression of HSPGs in polarized epithelial cells and revealed that glypican, unlike most other GPI-anchored proteins characterized to date, does not have an exclusive or even preferential apical localization. Glypican is expressed apically, and is in this sense unique amongst the cell surface proteoglycans (investigated here), but to a large extent it is also coexpressed with the transmembrane HSPGs (syndecan-1 and -4), which appear restricted to the basolateral surface domains of these cells. Glypican is also shed from these membrane compartments into both the apical and basolateral culture media. Yet, a glypican mutant that forms a poor substrate for the glycanation machinery accumulates nearly quantitatively in the apical culture compartments, suggesting that the HS chains interfere with the polarized expression of this protein. The physiological significance of the latter observation remains uncertain, but leads us to the formulation of two alternative hypotheses for further exploration. One is that substitution of GPI-linked core proteins with HS may represent a means of overriding the apical sorting signal conveyed by GPI anchors, and control the subcellular expression of these proteins in polarized systems. The other is that this interference represents a means of stabilizing or controlling the amount of HS and possibly of HS-associated ligands expressed at the apical surface of the cells. Modulation of the glycanation of glypican or of this interference could provide a basis for the variability of the subcellular expression of glypican that can be observed in situ.

Basolateral Expression of the Syndecans

Immunocytochemical experiments previously reported by Rapraeger et al. (1986) had shown that syndecan-1, initially present on the entire surface of the membrane of mouse mammary epithelial cells, ultimately becomes sequestered at the basolateral cell surface as the cells polarize in vitro. The present results of domain-specific biotinylation experiments on CaCo-2 cells, indicating that all transmembrane proteoglycans expressed by these cells are concentrated in the basolateral membrane compartment, extend this observation to other cell types and to other members of the syndecan family. Numerous experiments have shown that sequences in the cytoplasmic domains of proteins (e.g., polymeric immunoglobulin receptor, low density lipoprotein receptor) function as basolateral targeting signals (Hopkins, 1991), and studies on the expression of cytoplasmic deletion mutants of syndecan-1 (Mettienen et al., 1994) and syndecan-4 (our own unpublished observations) in MDCK cells suggest that this is also the case for the syndecans. Although no consensus sequence has been identified so far, at least in some cases these signals include a crucial tyrosine residue (Mostov et al., 1992). The cytoplasmic domains of the syndecans have 60–70%

of sequence identity, including the strict conservation of three tyrosine residues. It is possible that some of these residues function as a basolateral sorting signal, but this has not been shown directly. Since the syndecans can bind extracellular matrix components via their heparan sulfate chains and probably intracellular structures via their cytoplasmic domains, their confinement to the basolateral surface makes them prime candidates for anchoring cells to the underlying matrix and associated growth factors, but less or unsuitable for mediating heparan sulfate–dependent processes that involve apical reactants.

Bipolar Expression of Glypican

Similar investigations on the subcellular expressions of the members of the family of the glypiated HSPGs had not been reported so far. The present results indicate that glypican, which is expressed in many different cell types, accounts for most of the apical HS in CaCo-2 cells, and appear consistent with reports on the in situ localization of this proteoglycan in axonal projections that are thought to represent the equivalent of the apical compartments of polarized simple epithelia (Karthikeyan et al., 1994). Glypican, and the other members of this family possibly as well, represents therefore a logical candidate for mediating processes that involve apically exposed HS-dependent reactants (e.g., anticoagulation, lipoprotein lipase anchoring, recruitment of circulating cells). Usually, however, GPI-linked proteins are found exclusively on the apical surfaces of polarized epithelial cells, including MDCK and CaCo-2 cells (Lisanti and Rodriguez-Boulan, 1990; Lisanti et al., 1990). The unexpected aspect of the present results, therefore, is the evidence for a nonpolarized expression of the hydrophobic PLC-sensitive form of this proteoglycan in cells that express other endogenous GPI-linked proteins such as carcinoembryonic antigen (Garcia et al., 1991) exclusively at their apical surfaces. In the presence of confirmed correct sorting of reference GPI-linked proteins in these CaCo-2 cells, this finding and a similar aberrant polarization of this proteoglycan in transfectant MDCK cells (which in contrast to CaCo-2 cells use only direct apical sorting pathways) suggests that glypican does not obey "classical" sorting rules. Basolateral expressions of GPI-linked proteins have been reported before in a few other exceptional cases. At steady state, a small proportion (10%) of alkaline phosphatase can be detected at the basolateral side of CaCo-2 cells (Le Bivic et al., 1990). This fraction originates from a small pool of newly synthesized alkaline phosphatase that is missorted to the basolateral surface and inefficiently transcytosed. The indirect sorting pathway (basolateral delivery followed by transcytosis to the apical surface) has also been described for the GPI-linked 5′ nucleotidase in hepatocytes, cells that are generally accepted to have no direct transport pathways (Schell et al., 1992). Although these two GPI-linked proteins were detected at the basolateral membranes, their steady state levels at this site were much lower than what we found for glypican, as more than half of this proteoglycan accumulates in the basolateral compartments.

Glycanation Interferes with the GPI-sorting Signal

Glycolipid self-association into microdomains or "rafts" that are recognized by specific sorting proteins is thought to form the basis for the budding of an apical vesicle (Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990). GPI proteins are thought to associate with these rafts, whereas proteins with destinations for the basolateral membrane would be excluded from these structures. The finding that epithelial GPI-anchored proteins and glycosphingolipids are associated with complexes that are insoluble in cold solutions of detergents as TX-100, but efficiently extracted in cold solutions of octylglucoside (Brown and Rose, 1992), supports this model and TX-100 insolubility is taken as a measure of association with these rafts. The nonpolarized expression of glypican in both CaCo-2 and MDCK cells, together with an unusual TX-100 extractability as compared to other glypiated proteins (e.g., CEA), suggest that a glypican-specific characteristic interferes with the normal apical sorting mechanism for GPI-linked proteins. The results obtained with the glypican mutants, whereby the chainless form is nearly exclusively directed to the apical compartment and the proteoglycan forms remain essentially unpolarized, suggest that this characteristic consists in the presence of the HS chains. Proteoglycan forms of glypican may escape from clustering into these domains, for example, because they associate with a protein containing basolateral determinants that mediates their transfer to the basolateral membrane. In that case a direct participation of the HS chains in this association appears unlikely. The binding interactions of this glycosaminoglycan largely depend on its anionic properties or specific sequence composition and chlorate, which prevents HS sulfation, does not affect the polarity of the glypican expression. We cannot exclude, however, that removing the chains changes the configuration of the protein to an extent that protein-protein association might be prevented. The experiments with the syndecan-4/glypican chimera suggest, however, that most of the glypican protein is dispensable and that the presence of HS suffices to induce the randomization of GPI-anchored proteins. The findings seem more consistent with an exclusion model. Clustering of GPI anchors is thought to lead to very close protein packings, from which the proteoglycan form of the molecule may tend to be excluded. Studies on the sorting of GPI-linked proteins in a Fischer rat thyroid (FRT) cell line and a Con A^r mutant of MDCK cells, in addition indicate that clustering of the GPI anchor and glycosphingolipids is not sufficient for apical targeting, and that the interaction of GPI-linked molecules with VIP21/caveolin and additional adaptor proteins appear required for their apical sorting (Zurzolo et al., 1994). The HS chains in glypican are located near the carboxyl terminus of the core protein, close to the membrane and might prevent the interaction of glypican with such sorting components. To a certain extent such exclusion effects of the HS chains would be reminiscent of the function of the HS chains in the glomerular basement membrane, where they provide repellant-fixed negative charges that determine the selectivity barrier of the filtration apparatus. Finally, we can probably also exclude that glypican, at first, is sorted correctly to the apical surface followed by a HS dependent but chlorate-insensitive transcytosis to the basolateral surface. The short term labeling and the pulse–chase data do not support such a model.

Heparan Sulfate Effects on Sorting versus Sorting of Heparan Sulfate

Irrespective of the mechanism, the results obtained with the HS glypican mutants in MDCK cells represent one of the few examples of carbohydrate structures that can influence the polarity of the expression of a protein, and in this case of a glypiated protein. These findings imply that HS chains, which are important for many proteoglycan ligand binding interactions and functions, may also be physiological determinants of the location of these proteins in specific membrane compartments. Control over targeting signals would represent a novel function for these complex and versatile carbohydrates. In this context it may be relevant to recall that the GPI-linked HSPGs identified so far (glypican, cerebroglycan, the protein encoded by the OCI-5 transcript, K-glypican) share a unique cysteine motif and that the primary sequences of these proteins appear to be highly conserved, suggesting that these proteins may support essential functions by themselves (e.g., association with caveolar-signaling components). Modulation of the glycanation of these proteins may determine where these protein functions are expressed. In the case of glypican, a preliminary immunohistochemical survey of several tissues has indicated that the subcellular expression of this protein is quite variable, from near exclusive basolateral expressions to bipolar or pronounced apical localizations (Litwack et al., 1994; and our own unpublished results). In addition, for cerebroglycan, the neuronal glypican-related GPI-anchored HSPG, it has been noticed that the specific axonal localization of this proteoglycan, observed in vivo, is not reproduced when the neuronal cells are brought into culture, where it also decorates dendritic projections (Kumbasar, A., E. D. Litwack, C. S. Stipp, J. K. Ivins, A. Yang, and A. D. Lander. 1994. *Mol. Biol. Cell.* 55:303a). It is not known whether variable substitution of the glypican core occurs in vivo and therefore represents a potential steering mechanism, but studies on the effect of TGF-β on syndecan-1 (Rapraeger, 1989) and the existence of "part-time" proteoglycans such as CD44 and betaglycan suggest that the number of substituted attachment sites within a proteoglycan core protein may vary and that unsubstituted core proteins may be expressed. It is also clear that the length of the HS chains on a particular proteoglycan can vary depending on the cellular source or differentiation of the cell, and a survey of a large number of cell lines indicates that the glycanation of glypican is indeed quite variable (results not shown). Such variation may be due to synthetic differences or to processing by glycosidases (Yanagishita, 1992). From the present experiments we cannot make any predictions on the possible effects of variations in chain length rather than chain number on the sorting of glypican, since the average chain length was similar in all transfectants. We also observed no internal differences in HS chain length between apical and basolateral forms of glypican in the CaCo-2 cells (where the HS chains were shorter than in the transfectants) that could reflect such an effect (not shown).

On the other hand, the moderate but reproducible enhanced apical expressions of the two- and one-chain forms of glypican suggest the possibility that inverse glycanation-sorting relationships and variations in the HS content of glypican may result in the essentially nonpolarized expression of large proteoglycan forms and the apical polarization of less glycanated, more glycoprotein-like forms of the proteoglycan. Should this be the case under physiological conditions, one consequence might be that, in a cell which reduces its glycanation machinery, the apical compartment is less rapidly depleted of HS than the basolateral compartment. Calculations based on the data in Table III would indeed predict that, all other possible variables remaining constant (e.g., core protein production and chain length or structure), cells that produce two-chain instead of three-chain forms would have their apical membrane-associated HS levels nearly unchanged while severely reducing the levels associated with their basolateral membranes (Table III). Such an adaptation may allow a cell to maintain the activity of HS-dependent processes that occur in luminal compartments (e.g., anticoagulation) while processes that occur basolaterally (e.g., receptor activation) may be compromised. One other consideration, finally, is that conditions may occur where the chain interference is overruled. The proteoglycan form of glypican is "missorted" in the two cell types and under the conditions that were tested here, but there is no evidence that this a general or obligate phenomenon. Should there be ways to override the "exclusion" effect of the HS chains on the entry of glypican into normal GPI-sorting pathways (the binding of ligand?), then entry of glypican in this pathway may provide the means for cells to enhance the expression of HS and heparin-binding proteins in the apical compartment. In that case, it would seem worthwhile to test whether glypican might not function as a carrier in the transcytosis of heparin-binding proteins. Our data on CaCo-2 cells show that at least a fraction of the glypican that is expressed at the cell surface cycles between membrane compartments, which would be a minimal requirement for this possibility.

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