A PROTEOGLYCAN UNDERGOES DIFFERENT MODIFICATIONS EN ROUTE TO THE APICAL AND BASOLATERAL SURFACES OF MADIN-DARBY CANINE KIDNEY CELLS
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Running title: Proteoglycan modification in MDCK cells
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We have grown polarised epithelial Madin-Darby canine kidney II cells on
filters in the presence of ^35S-sulfate, ^3H-glucosamine or ^35S-cysteine/methionine
to study proteoglycan (PG) synthesis, sorting and secretion to the apical and
basolateral media. While most of the
^35S-sulfate label was recovered in
basolateral PGs, the ^3H-glucosamine
label was predominantly incorporated
into the glycosaminoglycan chains of
apical PGs, indicating that basolateral
PGs are more intensely sulfated than
their apical counterparts.
Expression of the PG serglycin with a
green fluorescent protein tag (SG-GFP)
in MDCK II cells produced a protein
core secreted 85 % apically, which was
largely modified by chondroitin sulfate
chains. Surprisingly, the 15 % of
secreted SG-GFP molecules recovered
basolaterally were more heavily sulfated
and displayed a different sulfation
pattern than the apical counterpart.
More detailed studies of the differential
modification of apically and
basolaterally secreted SG-GFP indicate
that the protein cores have been
designated to apical and basolateral
transport platforms before pathway-
specific post-translational modifications
have been completed.

INTRODUCTION

Sorting and transport of newly synthesized
glycoproteins and PGs. In these cells,
apical sorting may be mediated, directly or
indirectly (1), by several classes of
glycans; N-glycans (2,3), O-glycans of the
mucin type (4) and chondroitin sulfate
(CS) glycosaminoglycans (GAGs;5),
while most basolateral sorting signals
identified so far are localized to the
cyttoplasmic tails of transmembrane
proteins (6), although heparan sulfate (HS)
chains also might direct basolateral
transport (7).
PGs are proteins that are modified by long,
usually unbranched polysaccharides
(GAGs) which polymerise in the Golgi
apparatus, a process catalysed by enzymes
specific for synthesis of either
CS/dermatan sulfate (DS) or heparin/HS
chains (5). HS chains are polymers of
alternating N-acetyl glucosamine and
gluuronic acid (Glcnac-GlcA) units (8,9),
while CS chains are polymers of N-acetyl
galactosamine and glucuronic acid
(Galnac-GlcA) units (10,11).
Both HS/heparin and CS/DS synthesis
starts by sequential addition of four
sugars; xylose, galactose, galactose and
gluuronic acid onto a serine next to a
glycine in the protein core (Ser-Xyl-Gal-
Gal-GlcA-). This linker tetrascaccharide is
coupled by the same enzymes for the two
GAG types, while addition of the fifth
sugar determines whether a GAG chain
becomes HS/heparin or CS/DS (5).
What actually determines whether the
GAG chain becomes CS/DS or HS is not
fully understood. Both the protein core and
the linker tetrascaccharide could be of
importance. Repeated serine-glycine
motifs (ser-gly) (1), a nearby cluster of
acidic amino acids (2), and larger globular
domains (3) have been shown to promote
HS substitution on PG protein cores.
Phosphorylation of the linker

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tetrasaccharide has been observed for both HS and CS chains, while sulfation has only been shown for CS chains (4). Both GAG-types may be extensively modified by sulfation, HS by deacetylation, and HS and DS by epimerisation (8,10). Generally, polymerization of CS chains is thought to take place in the TGN, while HS synthesis is completed in the cisternae preceding the TGN (16-19). Synthesis and sulfation of the linker tetrasaccharide is an event that naturally occurs before HS and CS polymerization (20), and therefore earlier in the secretory pathway. Linker tetrasaccharide synthesis has been proposed to start at ER exit sites, or early in the Golgi apparatus, and is presumably completed in the cis- and/or medial-cisternae (5). We could show that PGs secreted into the basolateral medium carried GAG chains that were more intensely sulfated and had a different sulfation pattern than their apical counterparts. By following one particular PG, serglycin with a green fluorescent protein tag (SG-GFP), we demonstrated that both the linker region, HS-chains and CS-chains were modified differently on apically and basolaterally secreted SG-GFP, indicating that apical and basolateral routes are segregated early in the Golgi apparatus, before the TGN, which has been regarded as the major site of sorting of apical and basolateral components in epithelial MDCK cells.

EXPERIMENTAL PROCEDURES

Construction of plasmid- cDNA of serglycin was a gift from S.O. Kolset (Dept. of Nutrition Res, University of Oslo). Expand Long Template PCR System (Roche) with 5`-primer (5`-ATCGGAATTCATGATGCAGAAGCTACTCAAA-3`) and 3`-primer (5`-TTGCAACGTACGATGGATCCTAACA TAAAATCCTCTT-3`) was used to amplify, and clone by standard molecular biology techniques into EcoRI and BamHI restriction sites in pEGFP-N3 (Clontech), making sergly-pEGFP.

Cell culture and transfections- MDCK II cells, were grown in DMEM with 5% Fetal calf serum (PAA, Australia), 1% penicillium/streptomycin and L-glutamine (Biowhittaker, Belgium) at 37°C and 5% CO2 to 50-70 % confluency in 100 x 20 mm culture dishes (Sarstedt) and transfected with 4µg plasmid (sergly-pEGFP) and 12 µl Fugene 6 (Roche). After 72 hours, the cells were passed and 1 mg/ml G-418 (Duchefa, NL) was added for selection of transfected MDCK II cells (MDCK II’). Resistant colonies were screened by IP with polyclonal anti-GFP (Abcam) and protein-A-sepharose (Amersham) before SDS-PAGE. Several colonies were propagated, and one was used for the experiments presented.

Metabolic labelling- MDCK II and MDCK II’ (10^6) were seeded on 4.7 cm^2 filters (Costar 3412) and grown for four ds, before labelling with 0.3 µCi/ml 35S-Cys/met (PerkinElmer) using DMEM without cys and met (Sigma), 0.3 µCi/ml 35S-SO4 (PerkinElmer) using RPMI 1640 without sulfate (Gibco), or 0.2 µCi/ml 3H-Glc(Gibco) for 22-24 hours. Apical (1 ml) and basolateral (2 ml) media were collected, and cell fractions were lysed in 1 ml IP-lysis solution (1% NP-40, 50 mM Tris pH 7.5, 2 mM EDTA, 150 mM NaCl, 35 µg/ml PMSF) added protease inhibitor tablets (Complete, Mini, EDTA free Protease Inhibitor Cocktail Tablets, Roche). Macromolecules were visualized by 4-12 % or 4-20 % SDS-PAGE (BioRad) after Sephadex G-50 Fine chromatography (16).

Immune precipitation- After preclearing with 60 µl (50-50 slurry) of protein-A-sepharose (Amersham), for one h at 4°C, IP of apical and basolateral media was carried out with 1 µl anti-GFP/ml at 4°C overnight, before addition of 60 µl (50-50 slurry) protein-A-sepharose for 3 hours at 4°C. The beads were washed six times with IP-wash solution (1% NP-40, 50 mM Tris pH 7.4, 150 mM NaCl, 0.05% Triton X-100) with 1% BSA, and 4 times without BSA. Beads were treated or not for GAG degradation, added SDS-sample buffer (XT, BioRad) and run on 4-12% XT SDS-PAGE with MOPS buffer (BioRad).

GAG degradation treatments- Samples were divided into three equal volumes;
control, chondroitinase ABC (cABC, Seikagaku Corp., Tokyo, Japan) treated for CS degradation at 37°C overnight, and HNO2 treated for HS degradation (10 min at RT), as described (17).

To obtain protein cores with CS tetrasaccharides only, 175 mU of Chondroitinase AC II Arthro (cAC II, Seikagaku) was added to each sample, together with cABC buffer (pH 6) for three hours at 37°C.

**Gel filtration chromatography - GAGs**
GAGs were treated for degradation with cABC or HNO2. Treated and untreated samples were applied to a column (1 cm x 40 cm) of Sepharose Cl-6B (Amersham Biosciences), along with blue dextran and K2CrO4 as internal standards. Elution was performed with 0.15 M NaCl in 0.05 M Tris HCl buffer pH 8.0 and 0.1% Triton X-100, at a rate of 6 ml/h. Fractions of 1 ml were collected and analysed for radioactivity in a scintillation counter.

**Quantification of SG-GFP**
Gels were fixed, treated with amplify (Amersham), dried and exposed to PhosphorImager screens, scanned (Typhoon 9410 PhosphorImager, Amersham), and quantified by ImageQuant (Amersham), or subjected to autoradiography with Hyperfilm ECL (Amersham).

**Ion-exchange chromatography**
Radioactively labelled macromolecules from media (purified on Sephadex G-50 Fine columns) or precipitated SG-GFP secreted from MDCK II# cells were subjected to DEAE ion-exchange chromatography (Econo system, Bio-Rad). Samples were diluted in 1 ml of Buffer A (8 M urea, 0.02 M Bis-Tris, 0.1 M NaCl) and applied to a 3 ml DEAE Sephacel (Amersham Biosciences) column. After a 10 ml wash (buffer A), a linear gradient from buffer A to 60 % of buffer B (8 M urea, 0.02 M Bis-Tris, 1.15 M NaCl) was applied. Collected fractions (1 ml) and aliquots were counted in a scintillation counter.

**Extraction of CS chains from IP SG-GFP and digestion to disaccharides**
The isolation of GAG chains was performed according to Lin et al. (21). After IP of SG-GFP from both apical and basolateral media, the samples were treated with pronase (0.8 mg/ml) in 0.5 ml pronase buffer (50 mM Tris/HCl, pH 8.1 mM CaCl2, 1% Triton X-100) at 55 °C overnight with end-over-end mixing. Subsequently, 0.4 mg of pronase E from Sigma-Aldrich was added and the samples were incubated for three more hours. After inactivation by boiling and adjustment of the samples to 2 mM MgCl2, 12 mM endonuclease (benzonase) from Sigma-Aldrich was added. The samples were then incubated for 2 hours at 37 °C and after heat inactivation of the enzyme, adjusted to a final concentration of 0.1 M NaCl. Subsequently, the samples were centrifuged at 14.000 x g for 10 min. The GAG chains were desalted and purified by ion-exchange chromatography on 0.3 ml DEAE-Sepahcel columns. The gels were primed by washing with 2 M NH4HCO3 and loading buffer pH 8 (50 mM Tris/HCl, pH 8, 0.1 M NaCl, 0.1% Triton X-100). The supernatants from the digestions were applied and the columns were washed successively with loading buffer pH 8, washing buffer pH 4 (50 mM NaAc, pH 4, 0.1 M NaCl, 0.1% Triton X-100) and 0.2 M NH4HCO3. The GAG chains were eluted with 3 x 0.3 ml of 2 M NH4HCO3. The eluates were collected in microcentrifuge tubes and repeatedly freeze dried in a Maxi Dry Lyo (Heto) until pH of the samples were close to 7. The GAG pools were then digested with 150 mU of cABC in a final volume of 50 µl of 40 mM Tris acetat buffer, pH 8.0, with 0.01% BSA. The CS digestions were allowed to proceed over night at 37 °C. Each sample was then added 50 µl MilliQ water (Waters, USA), centrifuged and the enzyme heat inactivated by boiling. After a second centrifugation, the samples were diluted to 250 µl and were ready for analysis on ion-pair chromatography on reversed phase HPLC (RPIP-HPLC).
**Analysis of CS disaccharides**

Quantitative analysis of GAG chains and their sulfation patterns were performed by RPIP-HPLC (5) on a Luna 5 μ C18 (2) reversed phase column (4.6 x 150mm) from Phenomenex (USA) in acetonitrile (8.5%) and tetra-n-butylammonium hydrogen sulfate (1.2 mM; Fluka (USA)) by applying a stepwise gradient of NaCl from 1 to 53%. The flow rate was 1.1 ml/min and the fluorescent labeling reaction was performed by the addition of 2-cyanoacetamide (0.25%, Sigma (USA)) in NaOH (0.5%) at a flow rate of 0.35 ml/min. Signals were quantified against known amounts of standard disaccharides analyzed in parallel runs. The ΔCS disaccharide standards were from Sigma and Grampian Enzymes (Orkney, Scotland UK). The HPLC equipment (pump, autosampler and fluorescence detector were all purchased from Dionex (CA, USA), including the apparatus for online post-column delivery of solutions (PC10 Postcolumn Pneumatic Delivery Package). The chromatography software used was Chromeleon from Dionex.

**RESULTS**

PGs may be labelled in their protein cores by 35S-Cys/met, in the backbone of their GAG chains, by 3H-Glcn, or by 35S-sulfate when GAGs are modified by Golgi sulfotransferases. 35S-sulfate is the most specific metabolic label for PGs, since it is incorporated into other classes of glycoproteins to a lesser extent than the 3H-Glcn and 35S-amino acid labels. However, regardless the metabolic label chosen, PGs may be separated from other labeled molecules by ion-exchange chromatography, due to their high negative charge density.

We have previously demonstrated that MDCK cells synthesize several PG species (22), with either HS or CS chains, and that endogenous CSPG and hexyl-β-D-thiogalactoside-based CS chains are preferentially secreted apically (23). However, a thorough comparison of the GAG chains on apical and basolateral PGs has not yet been undertaken.

We therefore labeled filter-grown MDCK II cells metabolically with each of the three labeling agents, before total secreted macromolecules in the apical and basolateral media were isolated by gel filtration (Fig. 1 A-C) and 3H-Glcn and 35S-sulfate labeled macromolecules were loaded onto SDS-PAGE gels. Sulfated macromolecules were preferentially detected basolaterally, also in the PG region of the gels (Fig. 2, upper region, lane 7 and 10), in accordance with previous investigations.

Macromolecules labeled with 3H-Glcn were, on the other hand, mostly detected apically (Fig. 1 A), also in the PG region of the SDS-PAGE gels (Fig. 2, lane 1, and 4). The PG region of the gel split into two bands (Fig. 2, lane 1), where the upper band was sensitive to HNO2 treatment (Fig. 2, lane 2) and the lower band to cABC (Fig. 2, lane 3). To investigate further the reason for the discrepancy between the two labeling methods, 3H-Glcn labeled macromolecules from both media were subjected to DEAE ion-exchange chromatography. The apical medium contained a major PG peak eluting at 0.35-0.40 M NaCl, and a minor peak eluting at 0.55-0.6 M NaCl. For the basolateral medium the situation was the opposite, where the peak eluting at the higher salt concentration dominated (Fig. 3). This indicates that PGs secreted basolaterally are more charged than PGs secreted apically, and may explain some of the differences observed when comparing the incorporation of 35S-sulfate and 3H-Glcn.

These differences could be a result of alternative phenomena. Either, one or more PGs that are preferentially secreted at the basolateral membrane are more heavily sulfated than apical PGs, or there are general differences in apical and basolateral sulfation patterns of GAGs. To investigate these questions further we next wanted to study the post-translational modification, sorting and secretion of a single PG species. We therefore stably expressed serglycin with a C-terminal GFP tag (SG-GFP; Fig. 4 A) in MDCK II cells. The MDCK II clone selected for further
experiments (MDCK II) was metabolically labeled with 3H-GlcNAc before labelled macromolecules were harvested and subjected to preparative ion-exchange chromatography (Fig. 4B and C) as performed for wild type cells (Fig. 3). The transfected clone showed a similar pattern to the parental cell line, with a peak specific for the basolateral medium eluting in the higher salt region of the chromatogram (Fig. 4C). The indicated peaks (1-4) were subjected to c-ABC and nitrous acid treatment prior to SDS-PAGE (Fig. 4D). Peak 1 showed little sensitivity to the treatments, and does therefore neither contain HS, nor CS. Peak 2 in both chromatograms were sensitive to cABC, while peak 3/4 could be completely degraded by nitrous acid. This shows that peak 2 mainly contains CS and that peak 3/4 mainly contains HS.

We next immunoprecipitated the secreted PG (SG-GFP) from apical and basolateral media. The SG-GFP did not seem to contribute significantly to the CS peak (peak 2, Fig. 4B-D), since this has a higher molecular weight than that of SG-GFP (Figs. 5 and 7). After metabolic labelling with 35S-Cys/met, we found 85% of the SG-GFP in the apical medium (Fig. 5A, and 6A). Also 3H-GlcNAc labelled SG-GFP was preferentially recovered from the apical medium (67%; Fig. 5B, and 6B), while after labelling with 35S-sulfate, followed by IP, most of the label was recovered basolaterally (Fig. 5C, and 6C). Our results indicate that basolaterally secreted SG-GFP is significantly more sulfated than SG-GFP secreted into the apical medium (Fig. 6D).

Both apical and basolateral SG-GFP molecules are mainly modified by CS chains. Digestion with cABC revealed a band of the size of the SG-GFP protein core for samples from both media (Fig. 7A, lanes 4, and 7). The remaining hexasaccharides after cABC treatment of 35S-sulfate labelled SG-GFP were clearly more sulfated (1.9 times) for basolateral samples. Further digestion with cAC II demonstrated that also the linker tetrasaccharide was sulfated as described before (24, 25), and evidently, also in this case, basolateral SG-GFP contained more sulfate (1.3 times) than the apical counterpart (Fig. 7D), indicating that sulfation of the linker region of basolateral SG-GFP is 8 – 9 times more abundant than for apical SG-GFP. Human serglycin has 8 potential GAG attachment sites, and serglycin has previously been shown to exist as hybrids between CS and heparin/HS (26, 27). The cABC treatment did not digest all material in the PG region, indicating that some modification of SG-GFP with HS had taken place. HNO2-treatment, which digest HS chains, of 35S-Cys/met labelled samples did not produce any naked protein cores, but reduced the label in the PG region of the gels to some extent.

After CS or HS depolymerization it therefore seemed that HS chains are only found on SG-GFP CS/HS hybrids, where CS chains dominate and that these hybrids coexist with SG-GFP modified exclusively with CS (Fig. 7).

To our knowledge, this is the first report of a protein, for which a basolateral pool has acquired different post-translational modifications from that secreted apically. This difference could result from a sorting event in the TGN, where more negatively charged GAG chains would have a greater affinity for basolateral transport carriers. Chlorate treatment efficiently inhibits sulfation in MDCK II cells, without affecting GAG polymerization too much (25, 28). We therefore treated MDCK SG-GFP cells with chlorate (50 mM), and reduced the incorporation of sulfate by 98% (not shown), but SG-GFP sorting remained unchanged, indicating that high negative charge was not mediating incorporation into basolateral transport carriers (Fig. 8).

To investigate the differences in apical and basolateral sulfation of CS in more detail, we processed CS chains from apically and basolaterally secreted SG-GFP for HPLC analysis of sulfated disaccharides. Apical and basolateral media from 18 filters were collected and all secreted SG-GFP from the two medium compartments was isolated by IP. Apical sulfated CS disaccharides were mainly of ΔDi-4S type, but also some ΔDi-6S (Fig. 9) was detected, in contrast to basolateral CS which contained almost exclusively ΔDi-
6S (Fig. 9). The basolateral ΔDi-6S disaccharides were clearly more abundant than the sum of apical ΔDi-4S and ΔDi-6S disaccharides. In addition, the ratio of sulfated to non-sulfated disaccharides was higher for basolateral than for apical CS chains (Table 1). Regarding the fact that only 15% of the SG-GFP protein cores are secreted basolaterally, these analysis is in accordance with the other experimental data demonstrating that basolateral SG-GFP is much more sulfated than the apical counterpart. A minor fraction of the GAG chains attached to SG-GFP was shown to be of the HS type (Fig. 7). Also these much less abundant HS chains were differentially modified in the apical and basolateral secretory pathways, when analyzed by ion-exchange chromatography (Fig. 10). Clearly, the HS chains on basolateral SG-GFP carry on average higher charge density, and thus more sulfate groups, than their apical counterpart.

DISCUSSION
In this study we have used polarized MDCK cells transfected to express the proteoglycan serglycin-GFP (SG-GFP) to investigate differences in sulfation of PGs in the apical and basolateral secretory pathways. Indications that such differences exist were first observed in experiments we performed with wild-type MDCK II cells. Our subsequent studies with SG-GFP show that the differences in sulfation are not confined to polymerized CS chains, that are sulfated in the trans-Golgi network. Also HS chains, which are synthesized in Golgi cisternae, and the linker tetrasaccharide of CS chains, which is coupled together before the protein core leaves the cis region of the Golgi apparatus, carry much more sulfate in basolaterally secreted SG-GFP.

We suggest that different fractions of PG protein cores partition to particular apical and basolateral membrane platforms before reaching the TGN. We find an early onset of the formation of dedicated lipid domains the most plausible explanation for the observed phenomena. Support for this possibility comes from studies with mammalian and yeast cells that indicate that stabilized lipid domains may form early in the Golgi apparatus (29, 30) or already in the ER (31).

To our knowledge this is the first report of a protein core that undergoes different post-translational modification in the Golgi apparatus, depending on apical or basolateral destination. By using three different metabolic labels (35S-Cys/met, 3H-GlcN, and 35S-Sulfate), we have shown a much higher ratio of 35S-Sulfate to 3H-GlcN or 35S-Cys/met incorporation for basolateral than for apical SG-GFP, in full length CS-chains, in the CS linker region, and in HS chains. CS disaccharide analysis supported the quantitative differences observed for CS sulfation, and did in addition demonstrate that there also were qualitative differences in sulfation of CS chains on SG-GFP in the apical and basolateral secretory pathways.

Endogenous PGs like perlecan and versican are secreted to both sides of MDCK cell monolayers (22) and might therefore be modified differently in the apical and basolateral pathways. This could again reflect that GAG chains may have different biological roles in separate physiological compartments. Basolateral PGs contribute to the structure of the extracellular matrix, and to binding of growth factors at the basal side of epithelia. Such binding requires the presence of certain sulfation patterns in the GAG chains (8). On the other hand, too much sulfate on apical glycoconjugates (including GAGs) in epithelial tissues, is assumed to contribute to the pathogenesis in cystic fibrosis, by formation of new binding sites for infectious bacteria (33). Thus, it may be required that epithelial tissues stringently regulate the sulfation of PGs transported to the apical and basolateral surfaces, respectively.

Whether differential post-translational processing also could happen to other glycoproteins than PGs has to our knowledge not been reported. Few glycan structures have been studied in sufficient detail to decide on this possibility, although the overall pattern of N-glycosylation is similar for apical and
basolateral glycoproteins synthesized by MDCK cells (34). Further work is needed to gain knowledge on how the glycan polymerases and sulfotransferases in the Golgi apparatus are organized to produce the differences we observe in the structure of GAGs secreted from the apical and basolateral surfaces of epithelial MDCK cell monolayers.
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The abbreviations used are: cABC, Chondroitinase-ABC; CS, Chondroitin sulfate; Cys/met, Cysteine/Methionine; DMEM, Dulbecco’s modified Eagle’s medium, GAG, Glycosaminoglycan; GFP, Green fluorescent protein; Glcn, Glucosamine; HS, Heparan sulfate; IP, Immune precipitation MDCK, Madin-Darby canine kidney; PG, Proteoglycan; SG, serglycin.

FIGURE LEGENDS

FIG.1. Apical and basolateral secretion of macromolecules from MDCK II.
Radioactivity in macromolecules from medium aliquots from filter grown metabolically labelled MDCK II cells was after Sephadex G-50 Fine columns determined in a scintillation counter. Graphical representations of $^3$H-Glcn (A), $^{35}$S-Sulfate (B), and $^{35}$S-Cys/met (C) are shown. D: $^3$H-Glcn labelled apical and basolateral media were divided in three, followed by either cABC- and HNO$_2$ –treatment or no treatment. Untreated and treated samples were then loaded on to a Sepharose Cl-6B gel filtration column. The degraded products were collected, and the fractions were counted. Chromatogrammes were made for control, cABC- and HNO$_2$ –treated samples from both media. The graphical areas of CS and HS were calculated and the controls subtracted.

FIG. 2. Secreted macromolecules from MDCK II cells.
MDCK II cells grown on filters were metabolically labelled with $^3$H-Glcn and $^{35}$S-SO$_4^{2-}$ for 24 hs. Labelled macromolecules from apical and basolateral media were purified on Sephadex G-50 Fine columns. HNO$_2$- and cABC-treated and untreated controls (1/30 of each sample) were run on 4-20% SDS-PAGE.

FIG.3. Secreted negatively charged macromolecules from MDCK II.
Purified $^3$H-Glcn labelled samples from apical and basolateral media were loaded on DEAE ion-exchange columns. After washing, a linear salt gradient was applied. Radioactivity in collected fractions (1 ml) was measured in a scintillation counter. More negatively charged macromolecules elute in the later fractions.

FIG. 4. MDCK II cells expressing SG-GFP (MDCK II$^a$).
A: The domains of SG-GFP DNA. Amino acid (aa) 1 to 27: signal sequence, aa 94-111: eight serine-glycine motifs and one phenylalanine-glycine. PCR amplified serglycin cDNA without
stop codon (aa 1-148) was subcloned into the pEGFP expression vector, introducing 5 extra aas (159-163) in front of GFP. B-C: Filter grown MDCK II° cells were radiolabelled with ³H-Glcn. Apical and basolateral media were purified on Sephadex G-50 Fine columns and analyzed by DEAE ion-exchange chromatography as described for Fig. 3. D: From each peak marked by * in fig 4B and C, three fractions were collected and pooled, desalted on Sephadex G-50 Fine column and dried. The samples were solubilized, divided in three aliquots; untreated (Cnt), HNO₂ treated for HS degradation (H), and cABC treated for CS degradation (C) and loaded onto a 4-20% SDS-PAGE.

FIG. 5. **Secreted SG-GFP.**
Filter grown MDCK II° cells were radiolabelled with ³⁵S-Cys/met, ³H-Glcn or ³⁵S-SO₄²⁻. Media were harvested before IP with an anti-GFP antibody and loaded on to a 4-12% XT SDS-PAGE. Gels were dried and visualized by phosphoimaging or exposed to film.

FIG. 6. **Polarity of SG-GFP secretion.**
Quantification by ImageQuant of apical versus basolateral distribution as % of total secreted IP SG-GFP: ³⁵S-Cys/met (A), ³H-Glcn (B), or ³⁵S-SO₄²⁻ (C). Each column shows the average of 9-15 filters. Amount incorporated sulfate on SG-GFP (from C) related to incorporated ³⁵S-Cys/met (from A) or ³H-Glcn (from B) are presented in D.

FIG. 7. **Glycosaminoglycans on SG-GFP.**
MDCK II° cells were metabolically labelled with ³⁵S-Cys/met (A), ³H-Glcn (B), or ³⁵S-SO₄²⁻ (C), before harvest of apical and basolateral media. After IP of SG-GFP, the samples were divided in three equal parts. One part remained untreated (Cnt), one part was treated with HNO₂ for HS degradation (H), and one part was cABC-treated for CS degradation (C). To visualize sulfation of the linker tetrasaccharide, ³⁵S-SO₄²⁻ labelled SG-GFP was treated with cAC II after cABC treatment (D).

FIG. 8. **Secretion of chlorate treated SG-GFP.**
Filter grown MDCK II° cells were metabolically labelled with ³⁵S-Cys/met in the absence or presence of 50 mM chlorate, followed by harvesting, IP, 4-12 % SDS-PAGE, and quantification by ImageQuant.

FIG. 9. **Disaccharide analysis of CS on SG-GFP.**
IP SG-GFP from the apical and basolateral media of 18 filters with confluent MDCK II° cells incubated for 24 hs, were treated as described in experimental procedures. CS disaccharides after cABC treatment were separated on a RPIP-HPLC column. HPLC patterns of apical and basolateral CS disaccharides are shown. The arrows indicate where the disaccharide standards elute. The peaks that appear between ΔDi-0S and ΔDi-4S positions in the chromatogrammes are due to background also seen in runs with disaccharide standards (not shown).

FIG. 10. **Sulfation of HS on secreted SG-GFP.**
³H-Glcn labelled SG-GFP samples from apical and basolateral media were subjected to IP and cABC treatment. SG-GFP samples with remaining HS chains were loaded onto a DEAE ion-exchange column and eluted as described above (Figs 3 and 4). Collected fractions were counted for radioactivity in a scintillation counter. The elution profiles of apical and basolateral SG-GFP with only HS chains are shown.

Table 1. **CS disaccharides of SG-GFP.**
Disaccharide distribution of apical and basolateral SG-GFP CS chains extracted from HPLC chromatograms corresponding to fig 9 A and B.
Fig 2

|         | $^3$H-Glcn |         | $^{35}$S-SO$_4^{2-}$ |
|---------|------------|---------|----------------------|
|         | Api        | Baso    | Api                  | Baso                  |
| Cnt     | H          | C       | Cnt                  | H                     |
| 1       | 2          | 3       | 4                    | 5                     |
| 6       | 7          | 8       | 9                    | 10                    |
| 11      | 12         |         |                      |                       |
Fig 5

A  
\[ \text{S-Cys/met} \]

B  
\[ \text{H-Glc} \]

C  
\[ \text{S-SO}_4^{2-} \]

1 2 3 4 5 6
Fig 7
Fig8

**S-cys/met** - incorporation

[Bar chart showing incorporation percentages for different conditions]
|     | ΔDi-0S (%) | ΔDi-4S (%) | ΔDi-6S (%) |
|-----|------------|------------|------------|
| Api | 61         | 34         | 5          |
| Baso| 43         | 0          | 57         |
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