Characteristics and Structural Requirements of Apical Sorting of the Rat Growth Hormone through the O-Glycosylated Stalk Region of Intestinal Sucrase-isomaltase*

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The apical sorting of the small intestinal membrane glycoprotein sucrase-isomaltase (SI) depends on the presence of O-linked glycans and the transmembrane domain. Here, we investigate the role of O-glycans carried by the Ser/Thr-rich stalk region of SI as an apical sorting signal and evaluate the spatial requirements for an efficient recognition of this signal. Several hybrid proteins are generated comprising the unsorted and O-glycosylated protein, the rat growth hormone (rGH), fused to either the transmembrane domain of SI (GH-SI<sub>TM</sub>), or the transmembrane and the stalk domains (GH-SI<sub>S/TM</sub>). Both constructs are randomly distributed over the apical and basolateral membranes of MDCK cells indicating that neither the transmembrane domain nor the O-glycans are sufficient per se for an apical delivery. Only when a polyglycine spacer is inserted between the stalk region of SI and the luminal part of rGH in the GH-SI<sub>Gr/S/TM</sub> fusion protein does efficient apical sorting of an O-glycosylated protein as well as a time-dependent association with detergent-insoluble lipid microdomains occur. Obviously, the polyglycine spacer facilitates the accessibility of the O-glycans in GH-SI<sub>Gr/S/TM</sub> to a putative sorting receptor, whereas these glycans are inadequately recognized in GH-SI<sub>S/TM</sub>. We conclude that the O-glycans in the stalk region of SI act as an apical sorting signal within a sorting machinery that comprises at least a carbohydrate-binding protein and fulfills specific spatial requirements provided, for example by a polyglycine spacer in the context of rGH or the P-domain within the SI enzyme complex.

The polarity of epithelial cells is characterized by two functionally and structurally different plasma membrane domains, the apical and the basolateral. Separated by tight junctions, the two surfaces contain distinct compositions of proteins and lipids. The maintenance of polarity requires sorting as well as domain-specific retention of newly synthesized and recycling proteins (1, 2). Protein constituents are transported along the secretory pathway to the trans-Golgi network (TGN)<sup>1</sup> where they are sorted to either one of these two domains (3–5). Basolateral targeting generally depends upon the existence of a tyrosine-based cryptic signal or a di-leucine motif in the cytoplasmic tail of the sorted protein (1, 6). The apical delivery of membrane and secretory proteins is more complex and utilizes several types of signals suggesting the existence of multiple binding sites for apical signals in the sorting machinery. Glycolipid anchors direct proteins to the apical surface of several types of epithelial cells (7), apparently by associating in the TGN with detergent-insoluble membrane domains enriched in glycosphingolipids and cholesterol (8, 9). The transmembrane segments of influenza virus neuraminidase (NA) (10, 11) and hemagglutinin (HA) specify apical transport, and in the case of HA, several residues critical for this function have been identified (12). N-Linked oligosaccharides on some secreted proteins appear to specify apical transport (13), although this mechanism does not apply to all secreted proteins (14, 15) and has not been conclusively demonstrated for membrane-bound proteins (16, 17). O-Glycosylation is also critically important in the sorting event of some membrane glycoproteins (18). Specific inhibition of O-glycosylation of intestinal sucrase-isomaltase with benzyl-GalNAc (benzyl-N-acetyl-α-D-galactosaminide) as well as deletion of the potentially O-glycosylated Ser/Thr-rich stalk domain abolished the high fidelity of apical sorting of SI and resulted in a random transport of the protein to both membranes (19, 20). Likewise, a dominantly O-glycosylated domain juxtapose the membrane of the neurotrophin receptor is presumably implicated in its apical sorting (14, 21).

It is not obvious, however, from these observations whether O-glycans constitute the sorting signal per se, or they impose a particular folding determinant in the context of the actual sorting signal. Analysis of various deletion mutants of SI demonstrated that O-glycosylation and membrane anchoring of SI are required for the association of the enzyme with cholesterol and glycosphingolipid-rich lipid microdomains and subsequent apical sorting. Moreover, a possible role for an O-glycosylated Ser/Thr-rich stalk domain that immediately follows the membrane domain and belongs to the isomaltase subunit in the sorting of SI has emerged. However, SI comprises two strongly homologous subunits that are both O-glycosylated and a possible contribution of O-glycans in the sucrase subunit to the structure of a putative sorting signal cannot be excluded. The basic aim of this paper is to assess the requirements needed for an efficient sorting of apical proteins using the high polarized protein sucrase-isomaltase as a model. In particular, the role of O-glycans located in the stalk region of SI and the spatial constraints that influence an efficient recognition of these

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1 The abbreviations used are: TGN, trans-Golgi network; SI, sucrase isomaltase; GH, growth hormone; rGH, rat growth hormone; PAGE, polyacrylamide gel electrophoresis; benzyl-GalNAc, benzyl-N-acetyl-α-D-galactosaminide; endo H, endoglycosidase H; endo F, endoglycosidase F; DMEM, Dulbecco’s modified Eagle’s medium; MDCK, Madin-Darby canine kidney cells; TM, transmembrane; SR, stalk region; TX-100, Triton X-100; NA, neuraminidase; HA, hemagglutinin; ER, endoplasmic reticulum; TEMED, N,N,N’,N’-tetramethylethylenediamine.
structures through putative sorting elements have been investigated. Using chimeras of the non-polarized protein model, the rat GH, fused to the stalk transmembrane domains of SI we could show that O-glycosylation is absolutely required for apical sorting. However, specific spatial requirements should be fulfilled for an efficient recognition of these glycans by a putative carbohydrate-binding protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes, T4 DNA polymerase, ligase, endo-β-N-acetylglucosaminidase H (endo H), endo-β-N-acetylglucosaminidase F (endo F), and N-acetylglucosamined sulfonfluoride were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Streptomycin, penicillin, Dulbecco’s modified Eagle’s medium (DMEM), minimum essential medium, methionine-free DMEM, and fetal calf serum were purchased from Life Technologies, Inc. (Eggenstein, Germany). L-[35S]Redivue PRO-MIX (800 Ci/mmol) and protein A-Sepharose were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Acrylamide, ammonium persulfate, dithiothreitol, 2-mercaptoethanol, SDS, TEMED, and Triton X-100 were acquired from Carl Roth GmbH & Co. (Karlsruhe, Germany). DEAE-dextran, benzamidine, aprotonin, leupeptin, pepstatin, molecular weight standards for SDS-PAGE, and trypsin were purchased from Sigma (Deisenhofen, Germany). Polynulcosomal antibody (rabbit IgG) against the stalk region of SI, which cross-reacts with rat GH, was a generous gift of Dr. Sinha (22).

**Expression of Chimeras of the Rat Growth Hormone Fused to Various Domains of SI in MDCK Cells**—Hydrophilic rat GH is an Unglycosylated polypeptide that is randomly secreted in polarized MDCK cells from the apical as well as basolateral membranes (13). It could be therefore conveniently used as a reporter gene to analyze the role of specific putative sorting signals. Previous studies on a possible role of N-glycosylation in apical sorting have utilized mutants of this protein, which contained potential N-glycosylation sites (13). As such the trafficking randomness of the protein could be abolished and polarized secretion of the protein through the apical membrane has occurred. Obviously, N-glycans are implicated in the sorting event either directly being the sorting signal itself or indirectly by generating particular structural determinants in rat GH that constitute the sorting signal. Previous observations from our own work have provided ample evidence for a strong implication of O-glycans in the sorting of intestinal sucrase-isomaltase (18–20). We examined the role of O-glycosylation in the apical sorting event by making chimeras comprising unglycosylated rat GH as a reporter gene and the Ser/Thr-stalk domain of SI. We first generated two chimeras. In the first one the cleavable signal sequence of the type I protein rat GH was eliminated and replaced by the N-terminal transmembrane domain (TM) of the type II protein SI, which contains an uncleavable signal sequence (sequence H9262 CTAAAACAACAATTAAGGCA-3/H11032). This construct comprised the sequences Met1-Ala32 of SI and Leu27-Phe216 of rat GH and is denoted GH-SITM (Fig. 1). The second construct was designed to directly assess the role of the stalk domain of SI in sorting and comprised the transmembrane domain and the stalk region of SI (Met1-Ser20) fused to the Leu27-Phe216 sequence of rat GH (denoted GH-SIstalkTM; H9262-CTAGAAAGACCATGGCAGGTCCGGAATCAGAAGG-3/H11032). The generated construct comprised the sequences Met1-Ala32 of SI and Leu27-Phe216 of rat GH and is denoted GH-SITM (Fig. 1).

The resulting product was cloned as a HindIII-NotI fragment into pCDNA3 (Invitrogen, Groningen, The Netherlands) to generate pCDNA3-GHSIstkTM. Similarly, the pCDNA3-GHSIstTM encoding the SI cytoplasmic tail, transmembrane domain, and stalk region of SI (amino acids 1–60) fused to rat growth hormone without the signal sequence (sequence CTAAAACAACAATTAAGGCA-3/H11032) was generated using the oligonucleotides: SI5HindIII, 5'-AAGCTTCTATGAAAATAAGATGG-3'; cSIstalkGH, 5'-CATGGCAGGATGTGTTTATTAGCATT-ACCTGCCATGCCCTTGTCCA-3'; and cGHSIstTM, 5'-GGGGGCGCGCTAGAAGAACACAGGCTGGTTTATTAGCATT-ACCTGCCATGCCCTTGTCCA-3'.

For generation of the plasmid pCDNA3-GHSIstkTM, encoding eight consecutive glycines inserted between the stalk domain of SI and the stalk region of rat GH, an AccI site was introduced into pCDNA3-GHSIstkTM by site-directed mutagenesis according to the Quiqchange protocol (Stratagene, Amsterdam, The Netherlands) using the following oligonucleotides: AccCI5', 5'-CAATCTTCTTCTGATTCATTACCTGCCATGCCCTTGTCCA-3'; and AccCII5', 5'-CATGGCAGGATGTGTTTATTAGCATT-ACCTGCCATGCCCTTGTCCA-3'; and cGHSIstTM, 5'-GGGGGCGCGCTAGAAGAACACAGGCTGGTTTATTAGCATT-ACCTGCCATGCCCTTGTCCA-3'.

For the generation of plasmid pCDNA3-GHSTockTM, encoding eight consecutive glycines inserted between the stalk domain of SI and the stalk region of rat GH, an AccI site was introduced into pCDNA3-GHSIstkTM by site-directed mutagenesis according to the Quiqchange protocol (Stratagene, Amsterdam, The Netherlands) using the following oligonucleotides: 5'-CCGGGACCATGGCAGGTCCGGAATCAGAAGG-3'.

**Sorting of SI Stalk Region**

**FIG. 1. Schematic representation of SI-GH fusion protein constructs.** Structural features of SI are shown according to previous studies (23, 44). The constructs are type II membrane proteins (N35S/SIstTM) containing SI stalk region at residue 60 andrat GH, and for the constructs of rat GH, an AccI site was introduced into pCDNA3-GHSIstkTM by site-directed mutagenesis according to the Quiqchange protocol (Stratagene, Amsterdam, The Netherlands) using the following oligonucleotides: AccCI5', 5'-CAATCTTCTTCTGATTCATTACCTGCCATGCCCTTGTCCA-3'; and AccCII5', 5'-CATGGCAGGATGTGTTTATTAGCATT-ACCTGCCATGCCCTTGTCCA-3'; and cGHSIstTM, 5'-GGGGGCGCGCTAGAAGAACACAGGCTGGTTTATTAGCATT-ACCTGCCATGCCCTTGTCCA-3'.

The resulting product was cloned as a HindIII-NotI fragment into pCDNA3 (Invitrogen, Groningen, The Netherlands) to generate pCDNA3-GHSIstkTM. Similarly, the pCDNA3-GHSIstTM encoding the SI cytoplasmic tail, transmembrane domain, and stalk region of SI (amino acids 1–60) fused to rat growth hormone without the signal sequence (sequence CTAAAACAACAATTAAGGCA-3/H11032) was generated using the oligonucleotides: SI5HindIII, 5'-AAGCTTCTATGAAAATAAGATGG-3'; cSIstalkGH, 5'-CATGGCAGGATGTGTTTATTAGCATT-ACCTGCCATGCCCTTGTCCA-3'; and cGHSIstTM, 5'-GGGGGCGCGCTAGAAGAACACAGGCTGGTTTATTAGCATT-ACCTGCCATGCCCTTGTCCA-3'.

For the generation of plasmid pCDNA3-GHSIstkTM, encoding eight consecutive glycines inserted between the stalk domain of SI and the stalk region of rat GH, an AccI site was introduced into pCDNA3-GHSIstkTM by site-directed mutagenesis according to the Quiqchange protocol (Stratagene, Amsterdam, The Netherlands) using the following oligonucleotides: AccCI5', 5'-CAATCTTCTTCTGATTCATTACCTGCCATGCCCTTGTCCA-3'; and AccCII5', 5'-CATGGCAGGATGTGTTTATTAGCATT-ACCTGCCATGCCCTTGTCCA-3'; and cGHSIstTM, 5'-GGGGGCGCGCTAGAAGAACACAGGCTGGTTTATTAGCATT-ACCTGCCATGCCCTTGTCCA-3'.

For the generation of plasmid pCDNA3-GHSTockTM, encoding eight consecutive glycines inserted between the stalk domain of SI and the stalk region of rat GH, an AccI site was introduced into pCDNA3-GHSIstkTM by site-directed mutagenesis according to the Quiqchange protocol (Stratagene, Amsterdam, The Netherlands) using the following oligonucleotides: 5'-CCGGGACCATGGCAGGTCCGGAATCAGAAGG-3'.

**Cell Culture and Transfection**—Madin-Darby canine kidney (MDCK) cells (strain II) were maintained subconfluent in DMEM (Life Technologics, Inc., Egggenstein, Germany) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO2 incubator. Transfections were performed using a calcium phosphate-DNA precipitation procedure (27). To establish stably expressing cell lines, cells were selected with 0.4 mg/ml G418 (Life Technologies, Inc.) for 2 weeks after which individual clones were isolated and screened for expression of the chimeric protein with a polyclonal monkey anti-mouse GH antibody which cross-reacts with rat GH (22). For analysis of cell surface polarity, cells were grown on Transwell filters (24 mm, 0.4 μm) (Becton Dickinson GmbH, Heidelberg, Germany) for 1 week after confluence. For all other experiments, cells were grown in 100-mm culture dishes (Greiner GmbH, Frickenhausen, Germany).

**Biiosynthetic Labeling of Cells, Immunoprecipitation, and SDS-PAGE**—Stably transfected MDCK cells were biosynthetically labeled with 80 Ci of L-[35S]Redivue PRO-MIX (Amersham Pharmacia Biotech, Freiburg, Germany) as described by Naïm et al. (28) either continuous or by employing a pulse-chase protocol. Here, cells were pulse-labeled for 30 min and chased for different periods of time with cold methionine. The cells were solubilized for 20 min at 4°C with a Nonidet P-40 lysis buffer (1% Nonidet P-40, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) containing a mixture of protease inhibitors 1 μg/ml aprotinin, 30 μg/ml pepstatin, and 100 μg/ml leupeptin.
phlorizin hydrolase. Here, MDCK cells were labeled for 4 h with 100 μCi of ¹⁴C]methionine. The protein chimeras were immunoprecipitated from intact cells by addition of anti-mouse GH to either the apical or basolateral compartments for 2 h at 4 °C. After extensive washing to remove the excessive antibody, the cells were solubilized on the filters by the addition of TX-100 extracts of non-labeled transfected MDCK cells and the antigen-antibody complex was captured by protein A-Sepharose. The immunoprecipitates were analyzed by SDS-PAGE according to Laemmli (31). After electrophoresis the gels were fixed, soaked in 16% salicylic acid for signal amplification, and subjected to fluorography, or quantified using a phosphorimaging device (Bio-Rad, Munich, Germany).

Analysis of the Glycosylation Pattern of the Chimeras—The presence of N-linked glycans in the various protein chimeras was analyzed by treatment of the immunoprecipitated biosynthetically labeled proteins with endo H or endo F followed by SDS-PAGE on essentially according to Naim et al. (18). Following electrophoresis, the radioactive bands were visualized using a phosphorimaging device (Bio-Rad) or autoradiography. The presence of O-linked glycans in the proteins was examined by biosynthetic labeling MDCK cells expressing the various chimeras in the presence or absence of 6 mM benzyl-GalNAc (Sigma, Deisenhofen, Germany), an inhibitor of O-glycosylation (32) as described by Alfallah et al. (20).

Association of the rGH-SI Chimeras with Membrane Microdomains—The association of the various protein chimeras with sphingolipid/cholesterol-rich microdomains was assessed in detergent extractability assays using TX-100 essentially as described before (20). Here, the cells were subjected to a pulse-chase protocol and solubilized for 2 h at 4 °C in a lysis buffer containing 1% TX-100, 25 mM Tris-HCl, pH 8.0, 50 mM NaCl. The detergent extracts were centrifuged at 100,000 × g at 4 °C, and the supernatant or the detergent-soluble fraction was retained for immunoprecipitation. The detergent-insoluble proteins recovered in the pellet were dissolved by boiling in 1% SDS for 10 min. Thereafter, 10-fold of a buffer containing 1% TX-100 was added. These and the TX-100-soluble fraction were immunoprecipitated with the polyclonal anti-mouse GH that recognizes native and denatured forms of rat GH (22).

RESULTS

Biosynthesis and Processing of GH-SITM and GH-SISR/TM—MDCK cells expressing chimeric forms of rat GH and SI were subjected to a pulse-chase protocol followed by immunoprecipitation of detergent extracts of the labeled cells with anti-GH antibodies. Fig. 2A compares the various GH forms. GH-STM was found in the cell lysates as a 25-kDa polypeptide, and its molecular form did not alter during the chase. The culture medium was devoid of a comparable polypeptide confirming the membrane association of this chimera (not shown). The chimera comprising GH and the stalk and the transmembrane domains of SI, GH-SISR/TM, on the other hand revealed two biosynthetic forms, 28- and 54-kDa species, which displayed a precursor-product relationship. The 28-kDa polypeptide was revealed at early chase point and gradually disappeared with a concomitant appearance of the 54-kDa protein. The 28-kDa protein represents, therefore, the precursor form of GH-SISR/TM (pGH-SISR/TM) and the 54-kDa protein its final mature form (mGH-SISR/TM). The faint but definite mGH-SISR/TM band appearing already at an early chase point is reminiscent of rapid kinetics of processing of pGH-SISR/TM to mGH-SISR/TM. Wild type GH is neither N- nor O-glycosylated; the shift to a larger size of 34-kDa during chase can be, therefore, assigned exclusively to the presence of the Ser/Thr-rich stalk domain of SI and possible O-glycosylation of the chimeric protein. We therefore examined the glycosylation features of GH-SISR/TM using enzymatic treatments. As expected, neither endo H nor endo F, which have specificities in cleaving N-linked glycans, altered the electrophoretic pattern of pGH-SISR/TM or mGH-SISR/TM (Fig. 2B). We further corroborated these results by treating cells with benzyl-GalNAc, which specifically inhibits O-glycosylation. While the precursor pGH-SISR/TM was not affected upon benzyl-GalNAc treatment, a significant shift in the size of mGH-SISR/TM could be observed (Fig. 2C). Together, the results unequivocally demonstrate that the 34-kDa mGH-SISR/TM polypeptide is O-glycosylated whereas pGH-SISR/TM is not. By virtue of the fact that O-glycosylation occurs in the Golgi apparatus, it is obvious that the chimera GH-SISR/TM has at least egressed the ER to the Golgi. By contrast to GH-SISR/TM, the GH-SITM chimera did not undergo post-translational processing, because the earliest detectable form did not change throughout the pulse and chase periods. The amino acid sequence of GH-SITM does not reveal potential N- or O-glycosylation, and it is unlikely that this chimera is glycosylated. We confirmed this by employing deglycosylation analyses with endo H and endo F (Fig. 2B) and by inhibition of O-glycosylation by treatment of the MDCK cells with benzyl-GalNAc as described for GH-SISR/TM (Fig. 2C). These treatments did not show any shift in the apparent molecular weight of GH-SITM, therefore, excluding that this polypeptide is glycosylated.

Cell Surface Expression of GH-SISR/TM and GH-SISR/TM—The biosynthetic labeling experiments have provided unequivocal evidence for transport competence of GH-SISR/TM from the ER to the Golgi and an efficient processing of the pGH-SISR/TM form to mGH-SISR/TM. We investigated further the fate of GH-SISR/TM and its transport to the cell surface particularly emphasizing the polarized sorting of this chimera in MDCK cells and the possible role played by the stalk and transmembrane domains of SI in this event. With this in mind, MDCK cells expressing the chimera GH-SISR/TM were grown on transparent polyester membranes in multwell tissue culture plates to allow separate isolation of the apical and basolateral surface antigens by cell surface immunoprecipitation. Postconfluent cells were biosynthetically labeled, and the antigenic material expressed on either surface was immunoprecipitated by adding anti-GH antibody to the apical or basolateral compartments. The control employed the chimera, containing the transmembrane domain and lacking the stalk region GH-SITM. Fig. 2D shows that GH-SITM was almost equally distributed over the apical and basolateral membranes (55–45%) indicative of a default targeting of this chimera to either membrane. The presence of the stalk region in the chimera GH-SISR/TM increased the targeting of this chimera to the apical membrane to about 60%. The apical versus basolateral distribution of this chimera (60–40%) is comparable to the distribution of a number of proteins described as being targeted by default or are not sorted in MDCK cells (29, 33). Previous studies have demonstrated that O-glycosylation of pro-SI, particularly that of its stalk domain, is required for sorting of pro-SI to the apical membrane with high fidelity, with almost 90% of surface-expressed pro-SI being found at the apical surface (19). It appears at first glance that the O-glycosylated stalk domain in the GH-SISR/TM chimera is not sufficient for its efficient apical sorting. Presumably, O-glycans in the context of this chimera are not sufficiently accessible or exposed to putative sorting elements.

The sorting mechanism of pro-SI occurs through its association with glycosphingolipid- and cholesterol-rich membrane microdomains, and this association is mediated by O-glycans. GH is a soluble protein and is not associated with lipid microdomains during its entire life cycle. We examined whether inducing O-glycosylation in the GH-SISR/TM chimera results in its
association with lipid microdomains and analyzed the deter-
gent extractability of GH-SI<br>TM with TX-100 in pulse-chase experiments. Fig. 2E shows that both forms of GH-SI<br>SRTM, pGH-SI<br>SRTM as well as mGH-SI<br>SRTM, were detergent-
soluble and appeared in the supernatant fraction. The deter-
gent-insoluble pellet that contains sphingolipid- and chol-
ester-rich microdomains was devoid of either protein form at all chase time points. Therefore, in a sharp contrast with pro-SI, it appeared at this stage of investigation that, in the context of GH-SI<br>SRTM, O-glycans neither constitute a strong apical sorting signal nor do they mediate association of this chimera with detergent-insoluble membrane microdomains. One explanation is that a recognition element, a lectin-like protein for example, may be implicated in the sorting event and that O-glycans in the chimaera are not sufficiently acces-
sible to this molecule due to steric hindrance imposed by the globular part of GH.

Biosynthesis, Processing, and Cell Surface Expression of a
Chimeric Protein Containing a Glycine Spacer Inserted between
the Stalk Domain of SI and GH—Based on the hypothesis
above, we inserted a spacer region comprising eight glycines
between the SR of SI and GH, GH-SI<br>Gly/SR/TM, to examine
whether this additional space would improve the access of
O-glycans to a putative receptor and increase thus the apical
sorting fidelity. The glycine linker is expected to be a flexible
structure that does not assume a particular folding determi-
nant within the overall context of GH as predicted from algo-


### Fig. 2. Biosynthesis of GH-SI<br>TM or GH-SI<br>SRTM in MDCK cells.

A, MDCK cells stably expressing either GH-SI<br>TM or GH-SI<br>SRTM were
biosynthetically labeled for 30 min at 37 °C with [35S]methionine and
chased with 2.5 mM unlabeled methionine for the indicated times. The
cells were solubilized with 1% TX-100, and the fusion proteins were
immunoprecipitated from the detergent extracts with a polyclonal anti-
mouse GH antibody followed by SDS-PAGE on 12% slab gels and
fluorography. B, MDCK cells stably expressing GH-SI<br>TM or GH-SI<br>SRTM were biosynthetically labeled for 4 h at 37 °C with [35S]methionine and
processed for immunoprecipitation as in A. Immunoprecipitates were
divided into three parts, which were treated or not treated with endo H
or endo F/GF followed by SDS-PAGE. C, MDCK cells stably expressing
GH-SI<br>TM or GH-SI<br>SRTM were biosynthetically labeled for 4 h at 37 °C with [35S]methionine in the absence or presence of 6 mM benzyl-GalNAc and
further processed for immunoprecipitation and SDS-PAGE as in A.
D, MDCK cells stably expressing GH-SI<br>TM or GH-SI<br>SRTM were grown on filters for 7 days after confluence and labeled with [35S]methionine at
37 °C for 4 h. The fusion GH-SI proteins located at the cell surface were
immunoprecipitated from either the apical (A) or basolateral (B) mem-
brane. The apical/basolateral distribution was quantified by scanning of
the fluorograms using a phosphorimaging device (Bio-Rad). n = 3. E, MDCK cells stably expressing GH-SI<br>TM or GH-SI<br>SRTM were labeled at
37 °C with [35S]methionine for 30 min and chased with 2.5 mM unlabeled methionine for the indicated times. The cells were solubilized with 1% TX-100, and the SI-GH fusion proteins were immunoprecipi-
tated from the detergent-soluble (S) and detergent-insoluble pellet (P)
fractions. The immunoprecipitates were analyzed by SDS-PAGE on
12% slab gels.

Pulse-chase experiments with cell lines expressing GH-SI<br>Gly/
SRTM revealed after 30 min of pulse one band corresponding to
the expected 28.6 kDa of the unmodified earliest detectable precursor (denoted pGH-SI<br>Gly/SRTM) (Fig. 3A). This protein was rapidly chased into a 34-kDa polypeptide, the intensity of which reached a maximum after 2 h of chase and persisted at comparable levels throughout the remaining chase periods (this will be referred to as mature or mGH-SI<br>Gly/SRTM). Here again and as described above for the chimeras, GH-SI<br>TM and GH-SI<br>SRTM, the glycosylation pattern of GH-SI<br>Gly/SRTM was analyzed. As expected, endo H and endo F did not alter the
electrophoretic pattern of either form of GH-SI<br>Gly/SRTM indicating
that the chimera is not N-glycosylated (Fig. 3B). This result was corroborated by treatment of cells expressing GH-
SI<br>Gly/SRTM with the inhibitor of O-glycosylation benzyl-Gal-
NAc, which generated a shift in the size of mGH-SI<br>Gly/SRTM to
∼31–32 kDa pointing to the presence of O-glycans in this fusion protein (Fig. 3C). O-Glycosylation of GH-SI<br>Gly/SRTM indicates also that this chimera has at least reached the Golgi
apparatus (35). To examine cell surface expression and polarized sorting of GH-SI<br>Gly/SRTM, the MDCK cell line expres-
sing this chimera was grown on membrane filters and cell
surface immunoprecipitation was performed after biosynthetic
labeling. Fig. 3D demonstrates that mGH-SI<br>Gly/SRTM was
found at the cell surface and, more importantly, that its tar-
geting reached high fidelity levels, because almost 85% of this
chimera was localized at the apical surface. pGH-SI<br>Gly/SRTM
was exclusively recovered intracellularly. The sorting pattern
of mGH-SI<br>Gly/SRTM is comparable to that of wild type SI in
Caco-2 cells (36) or when expressed in MDCK cells (20). Obvi-
ously, the substantial increase in the apical transport of mGH-
SI<br>Gly/SRTM could be attributed to the presence of the glycine
spacer.

As shown above and in sharp contrast to pro-SI, O-glycosy-
lated mGH-SI<br>SRTM, i.e. the mutant lacking the glycine spacer,
neither enters into detergent-insoluble membrane microdomains nor is it sorted with high fidelity to the apical membrane. We asked whether the high sorting fidelity revealed after introduction of the glycine spacer in the GH-SI Gly/SR/TM chimera is associated with an alteration in the detergent solubility of GH-SI Gly/SR/TM with TX-100. Pulse-chase analysis revealed that mGH-SI Gly/SR/TM, the O-glycosylated form of GH-SI Gly/SR/TM, could be identified in the TX-100-insoluble fractions at all time points (Fig. 3E), whereas its earliest detectable form, the non-glycosylated pGH-SI Gly/SR/TM precursor, was not. The presence of the glycine spacer has therefore not only generated a highly sorted chimera GH-SI Gly/SR/TM but has concomitantly lead to the acquisition of this mutant to detergent insolubility as compared with the spacer-free GH-SI Gly/SR/TM fusion protein. We wanted therefore to determine whether the glycine spacer per se directly alters the detergent insolubility of mGH-SI Gly/SR/TM or, more reasonably, its effect is indirect in providing sufficient spatial requirements for the O-glycans in the SI SR to interact with putative cellular components implicated in the biosynthesis of the lipid microdomains.

One approach is to inhibit or to influence the O-glycosylation event and determine whether an alteration in the detergent extractability has occurred. GH-SI Gly/SR/TM-expressing MDCK cells were biosynthetically labeled in the presence or absence of 6 mM benzyl-GalNAc, and the chimeric protein was assayed for its O-glycosylation. Concomitantly, this form became entirely soluble in TX-100 and was exclusively retained in supernatant fraction. The internal control, the non-glycosylated pGH-SI Gly/SR/TM, was as expected not affected by benzyl-GalNAc, and its solubility with TX-100 remained also unchanged (Fig. 4A). Because the glycine spacer is not glycosylated, the observed effect could be exclusively...
attributed to the reduced O-glycosylation of GH-SI_{Gly/SR/TM}—which was ensued by benzyl-GalNAc. The results demonstrate that the lipid raft-associated sorting of SI requires the presence of the O-linked glycans of the stalk. Furthermore, the glycans need to be in an accessible context for sorting to take place, because GH-SI_{Gly/SR/TM}, but not GH-SI_{SR/TM}, was predominantly found at the apical membrane.

**Cell Surface Expression of GH-SI_{Gly/SR/TM} in Benzyl-GalNAc-treated MDCK Cells**—Finally, we wanted to determine whether the reduction in O-glycosylation and the resulting failure to associate with membrane microdomains had implications on the sorting behavior of GH-SI_{Gly/SR/TM}. To achieve this goal, MDCK cells expressing GH-SI_{Gly/SR/TM} were treated with benzyl-GalNAc on membrane filters and the sorting pattern of the fusion protein was analyzed by cell surface immunoprecipitation. Here a default targeting to the apical and basolateral membranes of this glycoform (55%:45%) was discerned when O-glycosylation was affected in the presence of benzyl-GalNAc, whereas the normally O-glycosylated species (control) was targeted predominantly to the apical membrane as shown above (Fig. 4B).

**DISCUSSION**

Recent observations have implicated O-linked glycans as possible apical sorting signals (14, 18–20). The selective delivery of intestinal SI to the apical membrane in polarized Caco-2 or MDCK cells could be attributed to O-glycosidically linked carbohydrates that function in concert with the transmembrane domain of SI in driving SI to associate with detergent-insoluble lipid microdomains (18–20). In particular, the heavily O-glycosylated stalk domain of SI appears to constitute the main apical targeting signal. These observations, however, could not exclude a possible function of other heavily O-glycosylated domains in the apical sorting event of SI. The major focus of this report is therefore to determine whether the O-glycosylated stalk domain and the transmembrane region are sufficient to target SI to the apical membrane and possess therefore the exclusive function as a sorting signal per se. One approach is to fuse this stalk and the transmembrane domain to an unglycosylated and unsorted secretory protein. The rat GH fulfills adequately these criteria, and a chimeric protein containing rat GH fused to the stalk and transmembrane domains of SI (GH-SI_{SR/TM}) has been therefore used to delineate the function of these two domains in the sorting process. The GH-SI_{SR/TM} is a transport-competent protein that traverses the secretory pathway rapidly. The two structural components TM and SR in GH-SI_{SR/TM} endow this protein with characteristics reminiscent of a membrane-bound and O-glycosylated protein, indicating a correct integration and processing of these two domains, respectively.

The transport and secretion of rat GH into the external milieu in polarized MDCK cells occurs randomly, indicating that rat GH does not harbor specific sorting signals. The conversion of rat GH into a membrane-bound protein by fusing the TM of SI to the globular domain (GH-SI_{TM}) also does not alter the sorting behavior, thus indicating that the TM of SI is not equipped with polarized sorting elements. On the other hand, the TM of SI is a critical component in the overall sorting event of SI, and its presence constitutes an absolute requirement for SI to associate with lipid rafts and to be ultimately sorted to the apical membrane (19). Nevertheless and unlike the TM of certain integral viral protein, such as the influenza HA (12, 37), neuraminidase (38), and the envelope glycoprotein of simian virus (39), the TM of SI alone is not sufficient to mediate apical targeting. In fact, an SI deletion mutant containing the TM domain, but lacking another structurally critical domain, the O-glycosylated stalk region, is sorted randomly in epithelial cells and does not acquire TX-100 detergent insolubility during its passage through the TGN (19). The information harbored in the TM of the HA and neuraminidase, for example, is presumably indirectly utilized for the apical sorting event. It is most likely essential for the acquisition of these proteins to a particular quaternary structure, such as trimers or tetramers, and these structures facilitate the association of these proteins with lipid rafts that function as vehicles to the apical membrane. In fact, certain mutations in the TM domain of HA strongly affect its folding properties, including trimerization and susceptibility to trypsin, and inhibit its association with lipid rafts and subsequent apical targeting (12). A similar potential role for the TM of SI cannot therefore be hypothesized, because SI maintains a monomeric structure throughout its life cycle. Within the SI protein, the TM domain functions in concert with another critical and essential region in mediating association of SI with lipid rafts and apical sorting. Like the TM domain, the heavily O-glycosylated stalk region (SR) is a necessary but not a sufficient component of the sorting mechanism. Given the primordial importance of the TM and SR domains in the context of SI apical sorting, it is surprising at first glance that these domains in the GH-SI_{SR/TM} chimera are not capable of routing this protein to the apical membrane and mediating its interaction with lipid rafts. The possibility of altered structural features in the SR and TM within the context of rat GH is unlikely to explain the altered sorting behavior. Heavy O-glycosylation of the stalk region in the GH-SI_{SR/TM} occurs and is expected to generate a rigid unfolded structure that would behave autonomously in SI as well as in GH-SI_{SR/TM}. Likewise, the successful anchorage of GH-SI_{SR/TM} in the membrane through the foreign TM of SI is reminiscent of a fulfillment of the structural requirements of a type II protein. Defective or incorrectly processed fused domains can therefore be excluded as an argument to explain the default sorting of GH-SI_{SR/TM}. The association of SI with lipid microdomains is known to directly implicate O-glycans of the SR (19, 20) and occurs probably through an interaction with a specific lectin-type protein (20) that recognizes these O-linked sugar chains. One likely explanation for the failure of GH-SI_{SR/TM} to associate with lipid microdomains or rafts lies in an inadequate recognition of the O-glycans in GH-SI_{SR/TM} by a putative lectin-receptor due to geometrical restrictions upstream of the SR in GH-SI_{SR/TM}. Such restrictions are presumably not found in the SI molecule. This idea is strongly supported by the data presented here on the processing and sorting pathway of the GH-SI_{SR/TM} mutant. In this chimera SR is extended by eight aliphatic glycine residues fused between the SR and the globular rat GH domain to possibly eliminate a hypothesized geometrical restriction and to ultimately provide a better accessibility of the O-glycan residues to a putative lectin-like receptor.

The inclusion of the glycine spacer in GH-SI_{SR/TM} remains without effects on the biosynthesis, processing, O-glycosylation, and transport kinetics of the chimera but generates dramatic effects on its interaction with lipid microdomains and subsequent polarized sorting. The glycine-containing GH-SI_{Gly/SR/TM} construct associates with lipid microdomains and is sorted to the apical membrane with high fidelity similar to that of the SI molecule (19, 20). The association of GH-SI_{Gly/SR/TM} and many apically sorted proteins with detergent-insoluble lipid microdomains is transient and is reduced or terminated upon delivery of the protein to the apical membrane. Also, a shift from random transport, as in the case of GH-SI_{SR/TM} (60% apical versus 40% basolateral), to a high sorting fidelity of the GH-SI_{Gly/SR/TM} chimera (85%) is not exclusively due to an association of only an additional proportion (in this case 25%) of the molecules with detergent-insoluble lipid microdomains.
The data presented here could therefore unequivocally underscore the necessity of a spatial environment such as that endowed by the glycine spacer for a sufficient exposure and subsequent recognition of O-glycans in the GH-SI<sub>Gly/SR/TM</sub>-<sub>Gly/</sub> domain, which contains the stalk, would be dowed by the SR are responsible for the apical sorting behavior, recognition site required for the sorting event to ensue. Normally unsorted secretory protein. These residues can be mentors for binding to a putative lectin-like receptor. It can be excluded that protein-folding determinants endowed by the SR are responsible for the apical sorting behavior, otherwise the GH-SISR/TM, which contains the stalk, would have been correctly sorted; however, this is not the case. Furthermore, O-linked glycans in the stalk regions of proteins are rigid autonomous structures that are not expected to influence other proteinaceous domains, and in fact, inhibition of O-glycosylation does not prevent the folding pattern as judged by its unchanged reactivity toward trypsin. Despite the primordial importance of the O-glycans, they are not sufficient per se to warrant a correct sorting of proteins to the apical membrane. It is rather the adequate recognition of the O-glycans that constitutes a critical criterion for sorting, and this requires auxiliary elements that ultimately ensure the high fidelity of this event. Along these lines, our data are reminiscent of the existence of a recognition machinery that at least comprises a carbohydrate-binding protein and requires specific spatial requirements provided by the P-domain in SI or a glycine spacer in the context of rat GH.

A direct interaction of O-glycans with glycolipid membrane components in immediate proximity can be excluded as a mechanism underlying apical sorting of O-glycosylated GH-SISR/TM or the glycine-spacer containing GH-SI<sub>Gly/SR/TM</sub> construct or their association with lipid rafts, otherwise the O-glycosylated GH-SISR/TM would have also been interacting with lipid rafts and sorted correctly. It should be noted that the common feature of proteins that utilize O-glycans for their apical sorting is the organization and clustering of these chains into rigid stalks. Perhaps this is an important criterion for the strength and functionality of O-glycans as a signal and may explain why O-glycosylation does not play a role in sorting when it is not extensive or sporadically distributed over several protein domains.

REFERENCES

1. Matter, K., and Mellman, I. (1994) "Curr. Opin. Cell Biol. 6, 545–554"
2. Rodriguez-Boulan, E., and Powell, S. K. (1992) "Annu. Rev. Cell Biol. 8, 395–427"
3. Matlin, K. S., and Simons, K. (1984) "J. Cell Biol. 99, 2131–2139"
4. Simons, K., and Wandinger-Ness, A. (1990) "Cell 62, 207–210"
5. Hara-Kuge, S., Ohkura, T., Seko, A., and Yamashita, K. (1999) "Glycobiology 9, 833–839"
6. Keller, P., and Simons, K. (1997) "J. Cell Sci. 110, 3001–3009"
7. Lisanti, M. P., and Rodriguez-Boulan, E. (1990) "Trends Biochem. Sci. 15, 113–118"
8. Simons, K., and Ikonen, E. (1997) "Nature 387, 569–572"
9. Kurzchalia, T. V., and Parton, R. G. (1999) "Curr. Opin. Cell. Biol. 11, 424–431"
10. Wiede, A. W., Avalos, R. T., Sanderson, C. M., and Nayak, D. P. (1996) "J. Virol. 70, 6508–6515"
11. Barman, S., and Nayak, D. P. (2000) "J. Virol. 74, 6538–6545"
12. Lin, S., Naim, H. Y., Rodriguez, A. C., and Roth, M. G. (1998) "J. Cell Biol. 142, 51–57"
13. Scheiffele, P., Peranen, J., and Simons, K. (1995) "Nature 378, 96–98"
14. Yeaman, C., Le Gall, A. H., Baldwin, A. N., Monlauzeur, L., Le Bivic, A., and Rodriguez-Boulan, E. (1997) "J. Cell Biol. 139, 929–940"
15. Marzolo, M. P., Bull, P., and Gonzalez, A. (1997) "Proc. Natl. Acad. Sci. U. S. A. 94, 1834–1839"
16. Gut, A., Kappeler, F., Hyka, N., Balda, M. S., Hauri, H. P., and Matter, K. (1998) "EMBO J. 17, 1919–1929"
17. Rodriguez-Boulan, E., and Gonzalez, A. (1999) "Trends Cell Biol. 9, 291–294"
18. Naim, H. Y., Joberty, G., Alafalah, M., and Jacob, R. (1999) "J. Biol. Chem. 274, 17961–17967"
19. Jacob, R., Alafalah, M., Grunberg, J., Obendorf, M., and Naim, H. Y. (2000) "J. Biol. Chem. 275, 6566–6572"
20. Alafalah, M., Jacob, R., Preuss, U., Zimmer, K. P., Naim, H., and Naim, H. Y. (1999) "Curr. Biol. 9, 593–596"
21. Monlauzeur, L., Breuza, L., and Le Bivic, A. (1998) "J. Biol. Chem. 273, 30263–30270"
22. Sinha, Y. N., Selby, F. W., Lewis, U. J., and VanderLaan, W. P. (1972) "Endocrinology 91, 784–792"
23. Hunziker, W., Spies, M., Semenza, G., and Lodish, H. F. (1986) "Cell 46, 227–234"
24. Guan, J. L., and Rose, J. K. (1984) "Cell 37, 779–787"
25. Guan, J. L., Machamer, C. E., and Rose, J. K. (1985) "Cell 42, 489–496"
26. Moeslein, C. E., Ouwendijk, J., Wittbroth, M., Wissehaar, H. A., Hauri, H. P., Giesel, L. A., Naim, H. Y., and Fransen, J. A. (1997) "J. Cell Sci. 110, 557–567"
27. Gorman, C. M., Lane, D. P., and Rigby, P. W. (1984) "Philos. Trans. R. Soc. Lond.-Biol. Sci. 307, 343–346"
28. Naim, H. Y., Lacey, S. W., Sambrook, J. F., and Gething, M. J. (1991) J. Biol. Chem. 266, 12313–12320
29. Thomas, D. C., Brewer, C. B., and Roth, M. G. (1993) J. Biol. Chem. 268, 3313–3320
30. Jacob, R., Brewer, C., Fransen, J. A., and Naim, H. Y. (1994) J. Biol. Chem. 269, 2712–2721
31. Laemmli, U. K. (1970) Nature 227, 680–685
32. Huet, G., Hennebicq-Reig, S., de Bolos, C., Ulloa, F., Lesuffleur, T., Barbat, A., Carriere, V., Kim, I., Real, F. X., Delannoy, P., and Zweibaum, A. (1998) J. Cell Biol. 141, 1311–1322
33. Benting, J. H., Rietveld, A. G., and Simons, K. (1999) J. Cell Biol. 146, 313–320
34. Cuff, J. A., and Barton, G. J. (2000) Proteins 40, 502–511
35. Roth, J. (1984) J. Cell Biol. 98, 399–406
36. Garcia, M., Mirre, C., Quaroni, A., Reggio, H., and Le Bivic, A. (1993) J. Cell Sci. 106, 1281–1290
37. Rindler, M. J., Ivanov, I. E., Plesken, H., Rodriguez-Boulan, E., and Sabatini, D. D. (1984) J. Cell Biol. 98, 1394–1319
38. Jones, L. V., Compans, R. W., Davis, A. R., Bos, T. J., and Nayak, D. P. (1985) Mol. Cell. Biol. 5, 2181–2189
39. Huang, X. F., Compans, R. W., Chen, S., Lamb, R. A., and Arvan, P. (1997) J. Biol. Chem. 272, 27598–27604
40. Fiedler, K., and Simons, K. (1996) J. Cell Sci. 109, 271–276
41. Thihaute, V., Blanck, O., Courgeot, J., Pachetti, C., Perrin, C., de Mascarel, A., and Miquelis, R. (1995) Endocrinology 132, 468–476
42. Hauri, H., Appenzeller, C., Kuhn, F., and Nufer, O. (2000) FERS Lett. 476, 32–37
43. Fullekrug, J., Scheiffele, P., and Simons, K. (1999) J. Cell Sci. 112, 2813–2821
44. Naim, H. Y., Sterchi, E. E., and Lentze, M. J. (1988) J. Biol. Chem. 263, 7242–7253

Sorting of SI Stalk Region
Characteristics and Structural Requirements of Apical Sorting of the Rat Growth Hormone through the O-Glycosylated Stalk Region of Intestinal Sucrase-isomaltase
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