Molecular Basis of Aberrant Apical Protein Transport in an Intestinal Enzyme Disorder*

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The impaired sorting profile to the apical membrane of human intestinal sucrase-isomaltase is the underlying cause in the pathogenesis of a novel phenotype of intestinal congenital sucrase-isomaltase deficiency. Molecular characterization of this novel phenotype reveals a point mutation in the coding region of the sucrase-isomaltase (SI) gene that results in an amino acid substitution of a glutamine by arginine at residue 117 of the isomaltase subunit. This substitution is located in a domain revealing features of a trefoil motif or a P-domain in immediate vicinity of the heavily O-glycosylated stalk domain. Expression of the mutant SI phenotype in epithelial Madin-Darby canine kidney cells reveals a randomly targeted SI protein to the apical and basolateral membranes confirming an exclusive role of the Q117R mutation in generating this phenotype. Unlike wild type SI, the mutant protein is completely extractable with Triton X-100 despite the presence of O-glycans that serve in the wild type protein as an apical sorting signal and are required for the association of SI with detergent-insoluble lipid microdomains. Obviously the O-glycans are not adequately recognized in the context of the mutant SI, most likely due to altered folding of the P-domain that ultimately affects the access of the O-glycans to a putative sorting element.

The composition and function of the plasma membrane of polarized cells are maintained by a complex intracellular traffic moving cell surface glycoproteins between organelles. This requires the recognition and sorting of different classes of proteins not only during biosynthesis, but also during distributive events to the diverse cellular compartments (1). Oftentimes altered and defective intracellular trafficking of proteins due to single point or deletion mutations in the coding region of the gene result in pathological disorders (2, 3). Investigating the molecular basis of naturally occurring mutant protein phenotypes in diseases constitutes therefore a powerful means to unravel the molecular mechanisms underlying intracellular protein transport and sorting. A small intestinal disorder directly associated with a folding mutant and defective intracellular protein transport is congenital sucrase-isomaltase deficiency (CSID). CSID is an autosomal recessive disease that is characterized by an absent sucrase activity within the sucrase-isomaltase (SI) enzyme complex, while the isomaltase activity can vary from absent to normal. The disease is clinically manifested as an osmotic-fermentative diarrhea upon ingestion of di- and oligosaccharides (4). Several phenotypes of the disease have been characterized on the basis of cellular mislocalization or aberrant function of the SI mutant protein. SI is a type II membrane-bound glycoprotein of the intestinal brush border comprising two strongly homologous subunits, sucrase and isomaltase (5–8). These two domains originate from a large polypeptide precursor, pro-SI, by tryptic cleavage occurring in the intestinal lumen and ultimately maintain a strong association through noncovalent ionic interactions (9, 10). This enzyme complex is a heavily N- and O-glycosylated protein (10). Particularly O-glycosylation is critical for targeting of SI to the apical membrane through direct association in detergent-insoluble lipid rafts (11).

In this paper we describe a novel phenotype of CSID and the corresponding mutation, which results in a random distribution of the SI protein at the apical and basolateral membrane. Here a point mutation, an adenine to glutamine at nucleotide 412 in the coding region of the isomaltase subunit, results in a substitution of glutamine to arginine at amino acid residue 117. Defects in polarized protein sorting implicated in the pathogenesis of diseases have been rarely observed, perhaps because such defects have lethal consequences during early stages of development. Abnormalities in protein trafficking in polycystic kidney disease and cystic fibrosis have been shown to be associated with mutations in the coding regions of the corresponding genes (3, 12). The mutation identified in this report, however, is to our knowledge the first of its kind, in which aberrant polarized sorting of an intestinal protein is disease-associated. By establishing the functional details of this mutation and its implication in the polarized sorting of SI, the structure and function of the sorting elements possibly interacting within the region harboring the mutation could be elucidated.

MATERIALS AND METHODS

Processing of Biopsy Samples—CSID in a 4-year-old patient with acidic diarrhea was suggested by a sucrose tolerance test while under total parenteral nutrition. Biopsy specimens were obtained and immediately frozen in liquid nitrogen for enzyme activity measurements and RNA preparation.

Isolation and Mutagenesis of SI cDNA—Cloning of the SI cDNA from the patient’s mucosal cells followed the same strategy as described

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¶ The abbreviations used are: CSID, congenital sucrase-isomaltase deficiency; SI, sucrase-isomaltase (all forms); pro-SI, uncleaved sucrase-isomaltase; ER, endoplasmic reticulum; mAb, monoclonal antibody; endo H, endoglycosidase H; MDCK, Madin-Darby canine kidney cells; benzyl-GalNAc, benzyl-N-acetyl-α-D-galactosaminide; PAGE, polyacrylamide gel electrophoresis.
Previously (13). Messenger RNA was isolated using Dynabeads® Oligo(dT) (Dynal, Oslo, Norway), and cDNA was synthesized with the First Strand cDNA Synthesis Kit® (Amersham Pharmacia Biotech, Freiburg, Germany) using random hexamer nucleotide primers. For each PCR reaction 1/10 (2 μl) of the reaction mixture was used as a template. PCR reactions were performed with the seven primer pairs published by Ouwendijk et al. (13). The negative controls underwent the same procedure, only cDNA template was excluded from each reaction mixture. The products of two independent PCR reactions were directly cloned into the pCR®-2.1 Vector (Invitrogen, Groningen, The Netherlands) and sequenced in both orientations with M13 universe and reverse primers. The sequence analysis revealed a single mutation A/G at position 412. For MDCK cells the complete SI cDNA in the plasmid vector pSG8 (pSHI) (14) was mutated at position 412 by oligonucleotide-directed mutagenesis with the QuikChange™ in vitro Mutagenesis System from Stratagene (Amsterdam, The Netherlands). The following oligonucleotides were used in this context: SI WT cDNA, 5′-TTG TTA TAA CGT TCG AGA CAT GAC AAC AAG-3′ and SI muti, 5′-GTT GTC ATG TCT CGA AGC TTA ACA CCA-3′. Mutations were confirmed by sequencing, and the plasmid obtained was denoted pSHI<sub>mut</sub>.

Transfection and Metabolic Labeling of MDCK Cells—MDCK cells were co-transfected with pSHI<sub>mut</sub> and the neomycin resistance vector pcDNA3 (Invitrogen, Groningen, The Netherlands) using the calcium phosphate procedure as described previously (15). Stable cell lines expressing wild type or mutant SI were selected by using 0.25 mg/ml geneticin and of a selection medium (Life Technologies, Inc.). Isozymes of the following hybridomas: HBB 1/691, HBB 2/614, HBB 3/705 (19) provided by Dr. H. P. Hauri (13). The negative controls underwent the same procedure, only cDNA template was excluded from each reaction mixture. The sequence analysis revealed a single mutation A/G at position 412 by oligonucleotide-directed mutagenesis with the QuikChange™ in vitro Mutagenesis System from Stratagene (Amsterdam, The Netherlands). The following oligonucleotides were used in this context: SI<sub>WT</sub> cDNA, 5′-TTG TTA TAA CGT TCG AGA CAT GAC AAC AAG-3′ and SI<sub>mut</sub>, 5′-GTT GTC ATG TCT CGA AGC TTA ACA CCA-3′. Mutations were confirmed by sequencing, and the plasmid obtained was denoted pSHI<sub>mut</sub>.

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tein with striking homologies to sucrase-isomaltase. These do-
mains have been shown to be implicated in protein-protein
interaction or lectin-like interactions, in the pathology of mu-
cous epithelia and in modulating cell growth (for a review, see
Ref. 26). To analyze the detailed function and the significance
of this mutation in this CSID phenotype, we expressed the
mutant SI cDNA in the epithelial cell line MDCK cells, which
do not express endogenous SI. This cell line is particularly
convenient for these analyses borne out by its successful appli-
cation in the heterologous expression of several intestinal pro-
teins, including SI, which revealed structural and functional
features as well polarized sorting behavior similar to their
endogenously expressed wild type counterparts in intestinal
epithelial cells (16). Biosynthesis and processing of wild type
and mutant SI in MDCK are shown in Fig. 2. Continuous
metabolic labeling of the cells containing the wild type SI
protein for 6 h revealed a 210-kDa mannose-rich polypep-
tide (SIh) that shifted upon reaction with endo H to a smaller size
and an endo H-resistant 245-kDa complex glycosylated mature
protein (SIc) (Fig. 2A, denoted WT). A similar band pattern was
also observed with the cells containing the mutant protein (Fig.
2A, denoted mutant). However, the proportion of the complex
glycosylated protein in the total synthesized and processed SI
protein was significantly less than that in its wild type coun-
terpart, suggesting a slower rate of processing of the mannose-
rich to the complex form. We therefore determined the trans-
port and processing kinetics of both wild type and mutant SI
employing a pulse-chase protocol. At early chase time points
wild type SI appears as a 210-kDa mannose-rich SIh polypep-
tide that is gradually converted with increasing chase periods
to the complex glycosylated SIc species (Fig. 2B, denoted WT).
A similar band pattern was also observed with the cells containing the mutant protein (Fig.
2A, denoted mutant). However, the proportion of the complex
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to the complex glycosylated SIc species (Fig. 2B, denoted WT).
Qualitatively a similar biosynthetic pattern could be observed
for the mutant SI protein exemplified by the mannose-rich and
complex glycosylated polypeptides (Fig. 2B, denoted mutant).
However, the t1/2 time for the conversion of the mannose-rich
mutant SI into a complex glycosylated form was 4.5 h as com-
pared with −1.5 h of the wild type protein (Fig. 2C) compatible

![Fig. 2. Expression of mutant SI cDNA in MDCK cells. A, stable
MDCK cells expressing wild type SI (indicated WT) or mutant SI
(indicated mutant) were biosynthetically labeled with [35S]methionine
for 6 h. The cell lysates were immunoprecipitated with mAb anti-SI,
and the immunoprecipitates were treated or not treated with endo H.
The samples were finally analyzed by SDS-PAGE on 6% slab gels and
autoradiography. SIh is the mannose-rich endo H-sensitive form, and
SIc is the complex glycosylated endo H-resistant form. B, stable MDCK
cells containing either wild type SI (WT) or mutant SI (mutant) were
pulse-labeled for 30 min with [35S]methionine followed by a chase with
cold methionine for the indicated time points. The cell lysates were
immunoprecipitated with mAb anti-SI and subjected to SDS-PAGE on
6% slab gels. SIc, mannose-rich SI; SIc, complex glycosylated SI. C,
Quantification of the gels shown in B. D, stable MDCK cells were
biosynthetically labeled for 4 h (WT) or 8 h (mutant) with [35S]methi-
onine in the presence or absence of the 4 mM benzyl-GalNAc, an inhib-
itor of O-glycosylation. Detergent extracts of the cells were immunopre-
cipitated with mAb anti-SI and analyzed by SDS-PAGE on 6% slab gels.

![Fig. 3. Polarized expression of mutant SI in MDCK cells. A, MDCK cells expressing wild type (WT) and mutant SI were grown on
transmembrane filters, labeled with [35S]methionine for 4 h followed by
cell-surface immunoprecipitation of SI from the apical (panel a) or
basolateral (panel b) membranes. The immunoprecipitates were sub-
jected to SDS-PAGE. B, quantification of the proportions of complex
glycosylated SI shown in A.](image-url)
with delayed transport kinetics of this mutant from the ER to the Golgi apparatus. Clearly the effects on the intracellular transport of the mutant SI are due to the Q117R substitution, which has most likely altered some of the structural features of SI, perhaps in the domain containing the mutation itself, and may result in an increased degradation of mutant SI. This could explain the substantial reduction in the activity of iso-

maltase and sucrase in the biopsy specimen as well as in transfected MDCK cells. The Q117R substitution itself does not generate a novel sequon for O-linked glycosylation (27) and at the same time does not conform with a putative O-glycosylation motif (28). This strongly suggests that potential alterations in mutant SI are not due to differences in the N-glycosylation pattern, which is often implicated in the folding pattern of membrane glycoproteins already during early stages of post-

translational processing in the ER (29, 30). The pattern of O-glycosylation is similar in both cases, since treatments of cells with benzyl-GalNAc, an inhibitor of O-glycosylation (11, 18), revealed reductions in the size of the wild type and the mutant SI forms reminiscent of comparable contents of O-
glycans in both protein forms (Fig. 2D).

The next step was to determine whether complex glycosy-
lated mutant SI is correctly targeted to the apical membrane or whether the mutation is associated with the generation of a randomly or missorted SI phenotype. For this MDCK cells expressing the mutant and wild type proteins were grown on membrane filters, and their biosynthesis and polarized trans-
port were followed by isolating the cell surface antigens from the apical or basolateral compartments with a mAb anti-SI. Fig. 3 shows that the wild type SI protein was predominantly found at the apical membrane with almost 90% sorting fidelity, confirming previous data (16). By contrast, the mutant protein followed a random pattern of transport to the cell surface, since it appeared almost equally at both sides of the membrane. The results demonstrate therefore that the mutation Q117R is respon-
sible for the generation of a mistargeted SI phenotype. This finding was at first glance surprising, since mutant SI similar to the wild type protein is O-glycosylated, and apical sorting of SI is known to take place through O-linked carbohydrates as a sorting signal and association of the protein with Triton X-100-insoluble cholesterol- and sphingolipids-rich membrane microdomains (11). The sorting mechanism of mut-

ant SI was therefore examined and compared with that of the wild type protein. As shown in Fig. 4 a proportion of wild type SI was recovered in the Triton X-100-insoluble pellet, while the mutant SI protein was exclusively recovered in the detergent-
soluble phase. Mutant SI therefore does not associate with lipid microdomains despite the presence of O-glycans that are re-

quired for this association and for apical sorting. The fact that the mutation is not directly implicated in O-glycosylation strongly suggests that a protein structure implicated in the sorting process has been altered. A Ser/Thr-rich stalk region immediately upstream of the transmembrane domain of SI is heavily O-glycosylated and is required for the association of SI with lipid rafts (16). Deletion of this domain eliminates almost completely O-glycosylation of SI, thus affecting its detergent extractability concomitant with loss of its sorting fidelity, while the transport competence of SI per se remains unchanged. As shown here, the mutation Q117R induces similar effects with respect to detergent solubility and sorting of SI as the deletion mutants do. Unlike the deletion mutants, however, mutant SI is extensively O-glycosylated, but it is possible that these chains are not adequately recognized by a putative sorting element, a lectin-like protein for example. A recognition and subsequent binding would trigger the sequence of events lead-

ing to association of SI with lipid microdomains and its delivery to the apical membrane. The Q117R mutation is located in the P-domain in the immediate vicinity of the O-glycosylated stalk domain (24). It is likely that structural alterations in this domain have partially or completely masked the O-linked car-
bohydrates and thus hampered their recognition. Along this concept a correctly folded P-domain would be implicated in the sorting event by providing ample spatial requirements re-

quired for an adequate recognition of the O-glycosylated sort-
ing signal by a specific sorting protein. The existence, identity, and structure of components of the apical sorting machinery, in particular that of a putative cellular lectin-like protein specific for binding residues comprising O-linked chains, such as ga-

 lactose or N-acetylgalactosamine, are far from being unrav-

elled. It has been proposed that lectin-like proteins in trans-

Golgi network and post-Golgi compartments may recognize N-linked chains that function as apical sorting signals on some proteins and thus mediate their incorporation into apical car-

rier vesicles (31). VIP 36 is one of these candidates that binds specifically galactose and N-acetylgalactosamine residues (32) and can therefore function as a receptor for both N- and O-

linked glycans. Its detection, however, in basolateral carriers has raised questions as to its specific role in apical sorting. Nevertheless, some apical proteins are targeted to the apical membrane by transcytosis through the basolateral membrane and would also be transiently located in basolateral vesicles (33).

In summary, the first identification of a naturally occurring mutation responsible for random transport of an otherwise highly polarized protein of the brush border membrane pro-
vides a strong indication for the existence of a receptor protein that is required to recognize a sorting signal. The fact that the available O-linked glycans in mutant SI are not recognized by such a putative receptor suggests that its conformation should properly fit in the context of three structures of the SI protein, the P-domain, the O-glycosylated stalk region, and the transmembrane domain, which has been assigned a decisive role in the sorting pathway of SI (16). Strategies could therefore be designed to discover this putative protein by utilizing the SI protein and its mutant phenotype as promising models.

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