Altered Folding, Turnover, and Polarized Sorting Act in Concert to Define a Novel Pathomechanism of Congenital Sucrase-Isomaltase Deficiency*

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Naturally occurring mutants of membrane and secretory proteins are often associated with the pathogenesis of human diseases. Here, we describe the molecular basis of a novel phenotype of congenital sucrase-isomaltase deficiency (CSID), a disaccharide malabsorption disorder of the human intestine in which several structural features and functional capacities of the brush-border enzyme complex sucrase-isomaltase (SI) are affected. The cDNA encoding SI from a patient with CSID reveals a mutation in the isomaltase subunit of SI that results in the substitution of a cysteine by an arginine at amino acid residue 635 (C635R). When this mutation is introduced into the wild type cDNA of SI a mutant enzyme, SI(C635R), is generated that shows a predominant localization in the endoplasmic reticulum. Nevertheless, a definite localization of SI(C635R) in the Golgi apparatus and at the cell surface could be also observed. Epitope mapping with conformation-specific mAbs protease sensitivity assays, and enzymatic activity measurements demonstrate an altered folding pattern of SI(C635R) that is responsible for a substantially increased turnover rate and an aberrant sorting profile. Thus, SI(C635R) becomes distributed also at the basolateral membrane in contrast to wild type SI. Concomitant with the altered sorting pattern, the partial detergent extractability of wild type SI shifts to a complete detergent solubility with Triton X-100. The mutation has therefore affected an epitope responsible for the apical targeting fidelity of SI. Altogether, the combined effects of the C635R mutation on the turnover rate, function, polarized sorting, and detergent solubility of SI constitute a unique and novel pathomechanism of CSID.

The utilization of misfolding-related diseases has proven to be invaluable in dissection of the molecular mechanisms of protein transport and has unraveled several intriguing cell biological phenomena, such as in cases of congenital sucrase-isomaltase deficiency (CSID).2 This intestinal autosomal recessive disorder is characterized by the absence of the sucrase and most of the maltase digestive activity within the sucrase-isomaltase (SI) enzyme complex. The isomaltase activity varies from absent to normal (1). Clinically, the disease is manifested as an osmotic fermentative diarrhea upon ingestion of disaccharides and oligosaccharides (2). Analysis of this disorder at the molecular and subcellular levels has unraveled a number of phenotypes of CSID, which are characterized by perturbations in the intracellular transport, polarized sorting, aberrant processing, and defective function of SI (3–5). A few examples have been also reported, in which the misfolded protein products may escape the quality control system, instead of being either degraded or retained in the ER. A mutation at the position 620 of SI (L620P), for example, has been identified as one of the possible genetic modifications occurring in the CSID (6). Although this mutant is mainly found to be localized in the ER, it can be at least partially expressed also on the cell surface. Moreover, Propsting et al. (7) reported on the Q1098P mutation of SI causing an incomplete folding of the protein, which is however insufficient to trigger the complete protein block into the ER. In fact, the protein can exit the ER and reach the cis-Golgi compartment eluding partially the quality control in the ER.

SI is a type II transmembrane glycoprotein of two highly homologous subunits, sucrase and isomaltase (5, 8), that is expressed at the intestine brush-border membrane, where it serves as a catalyst for the cleavage of sugar and starch (9, 10). The two subunits are generated from a single polypeptide precursor, pro-SI, by trypsin cleavage in the intestinal lumen. The initial mannose-rich glycosylated pro-SI form exits the ER to the Golgi apparatus at a relatively slow rate (t1/2 ~ 65 min) and is processed to a complex glycosylated mature protein that is heavily N- and O-glycosylated (11). Particularly O-glycosylation is essential for the targeting of the protein to the apical membrane (12). In fact, O-glycans that are located in the Ser/Thr-rich stalk domain proximal to the transmembrane region constitute an essential component of the apical sorting signal of SI that functions through direct association with detergent-resistant membranes (DRMs) (13).

In this paper, we describe a new variant of CSID characterized by an exchange of a cysteine by an arginine at position 635 of the isomaltase subunit (C635R). We demonstrate that this mutant SI is transported to the cell surface at a substantially slower rate than its wild type counterpart, primarily because of an initial block in the ER. Moreover, an increased turnover, a reduced enzymatic activity, as well as partial mis-sorting of the mutant to the basolateral membrane altogether account for the generation of CSID phenotype.

MATERIALS AND METHODS

Antibodies—Four epitope-specific anti-SI monoclonal antibodies (mAb), obtained from the hybridoma cell lines HBB 1/691, HBB 2/614, HBB 2/219, and HBB 3/705 (14) and anti-lactase-phlorizin hydrolase and anti-aminopeptidase N mAbs were provided by H.P. Hauri (Biocenter, Basel, Switzerland) and E.E. Sterchi (University of Bern, Bern, Switzerland).

Processing of Biopsy Samples—Intestinal biopsy specimens were obtained from a 20-year-old male patient. Pieces of comparable size...
from the same tissue were used for RNA isolation and biosynthetic labeling of the endogenous SI in organ culture according to Naim et al. (15). The biopsy specimens were continuously labeled for 4 h with 100 μCi of L-[35S]methionine (>1000 Ci/mmol, Amersham Biosciences) in the presence of 3.5 ml of methionine-free medium, homogenized, and processed to immunoprecipitate SI as well as lactase-phlorizin hydrolyase and aminopeptidase N as control proteins (data not shown).

Cloning and Mutagenesis of a cDNA Encoding SI—Total mRNA was isolated from biopsy specimens by using the RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The cDNA was synthesized by using the First Strand cDNA Synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) with random hexamer nucleotide primers. The cDNA obtained from the patient was compared with the cDNA encoding the wild type SI by PCR. Seven different pairs of primers (16) were used to amplify different fragments of the SI-cDNA in the presence of Isis-DNA polymerase (Qbiogene, Heidelberg, Germany) having 3′-5′-exonuclease activity. Sequencing of the PCR products revealed a T/C exchange at the nucleotide 1903 corresponding to substitution of the cysteine 635 by an arginine in the isomaltase subunit. This mutation was introduced by site-directed mutagenesis into the wild type SI cloned in the vector pSG8-SI (17). The SI mutant will be denoted hereafter as SI(C635R). For confocal microscopic analyses of the mutation, the pEYPF-SI (18) was used. This vector encodes the chimeric form of wild type SI fused to the yellow fluorescent protein (YFP). The YFP-chimeras used in this study are denoted hereafter as SI-YFP (wild type) and SI(C635R)-YFP (mutant). They were obtained by PCR using the following primers: 5′-GGAATACCTTTGGTTGGAGCAGATATCC-3′ and 5′-GGTTTTCAGCACAACATCCACCGGATATCCTGCTCAACCAAAGGTATTC-3′. Synthesis of oligonucleotides and DNA sequencing was performed by MWG (Ebersberg, Germany).

Transfection of COS-1 and MDCK-II Cells—COS-1 cells were transiently transfected by the DEAE-dextran method as described previously by Naim et al. (15).

For the stable expression of SI-YFP and SI(C635R)-YFP in polarized cells, MDCK-II cells were transfected with Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Transfected cells were selected in Dulbecco’s modified Eagle’s medium (supplemented with 10% fetal bovine serum and 2 mM L-glutamine) containing 500 μg/ml Geneticin (Invitrogen) for 21 days.

Biosynthetic Labeling of COS-1 Cells, Immunoprecipitation, and SDS-PAGE—Transiently transfected COS-1 cells, expressing either wild type SI or SI(C635R), were labeled with 100 μCi of L-[35S]methionine in the presence of 3.5 ml of methionine-free medium. In pulse-chase experiments, after pulse labeling for 30 min to 2 h at 37 °C, cells were incubated in complete Dulbecco’s modified Eagle’s medium for different time intervals. SI was immunoprecipitated from cell lysates by using a combination of anti-SI mAb and protein A-Sepharose (Amersham Biosciences) (19, 20). Proteins were finally detected by using a phosphorimager (Amersham Biosciences). A postnuclear supernatant of the detergent extracts was prepared from the same tissue used for RNA isolation and biosynthetic labeling of the endogenous SI in organ culture according to Naim et al. (15). The biopsy specimens were continuously labeled for 4 h with 100 μCi of L-[35S]methionine (>1000 Ci/mmol, Amersham Biosciences) in the presence of 3.5 ml of methionine-free medium, homogenized, and processed to immunoprecipitate SI as well as lactase-phlorizin hydrolyase and aminopeptidase N as control proteins (data not shown).

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Transfection of COS-1 cells and of MDCK-II stable cell lines was performed with a Leica TCS SP2 microscope and a ×63 water planapochromat lens (Leica Microsystems, Bensheim, Germany). For indirect immunofluorescence, COS-1 cells expressing wild type SI or mutant SI(C635R) were fixed with 4% paraformaldehyde. The expression of SI proteins at the cell surface was analyzed by using a FITC secondary antibody conjugated with Alexa Fluor 488.

RESULTS

Biosynthesis and Processing of SI in the Cells of the Patient—The biosynthetic features of SI were first investigated in intestinal biopsy specimens obtained from a patient with CSID. The control utilized intestinal tissue from an individual who was biopsied for routine diagnostic purposes and most importantly did not suffer from malabsorption caused by enzyme deficiencies. The tissue specimens were labeled for 4 h with [35S]methionine, and SI was detected by immunoprecipitation from the

![](image)

**TABLE 1** Comparison of an amino acid stretch encompassing the mutation C635R

An alignment of the amino acids 628–647 of the SI from different species shows a high conservation of cysteine at position 635, indicating an important function of this domain.

C635R

| Species | Amino Acid | Cysteine |
|---------|------------|----------|
| WT      | PLVGAD1R   | C        |
| SI (human) | PLVGAD1C   | C        |
| SI (rabbit) | PLVGAD1C   | C        |
| SI (mouse)  | PLVGAD1C   | C        |
| SI (rat)    | PLVGAD1C   | C        |

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of trypsin for 15 or 60 min, respectively. The reaction was stopped by addition of 250 μg of soybean trypsin inhibitor.

Preparation of Detergent-resistant Microdomains—DRMs were essentially prepared according to Al-Oiah et al. (13). In brief, transfected COS-1 cells were biosynthetically labeled with 100 μCi of L-[35S]methionine for 4 h and lysed for 2 h on ice with 1% Triton X-100 in phosphate-buffered saline. A postnuclear supernatant of the detergent extracts was ultracentrifuged for 90 min at 100,000 × g. The supernatant and pellet fractions obtained were separately immunoprecipitated with anti-SI mAb and analyzed by SDS-PAGE (20).

Confocal Fluorescence Microscopy—Confocal laser microscopy of COS-1 cells and of MDCK-II stable cell lines was performed with a Leica TCS SP2 microscope and a ×63 water planapochromat lens (Leica Microsystems, Bensheim, Germany). For indirect immunofluorescence, COS-1 cells expressing wild type SI or mutant SI(C635R) were fixed with 4% paraformaldehyde. The expression of SI proteins at the cell surface was analyzed using an anti-SI mAb followed by an anti-mouse secondary antibody conjugated with Alexa Fluor 488.
total cell lysates. Fig. 1A shows the band pattern of the precursor forms of SI (pro-SI) expressed in the specimen of the patient and that of a control individual. The relative intensity of the mannose-rich (SI<sub>h</sub>) versus the complex glycosylated (SI<sub>c</sub>) form of pro-SI differs significantly in the tissue from the patient as compared with the control sample. Here, the ratio of the mature pro-SI to the ER-located mannose-rich protein is severalfold lower than that in the wild type counterpart. This could reflect substantial differences in the conversion rates of the immature pro-SI into its complex glycosylated counterpart in the patient affected by CSID.

Identification of an Amino Acid Substitution C635R and Assessment of Its Function—Previous reports have shown that point mutations in the coding region of the SI gene are responsible for misfolding and alteration in the trafficking of SI along the secretory pathway (5, 6, 16, 23, 24). We therefore isolated the cDNA encoding SI from the biopsy specimen of the patient and analyzed its sequence in comparison to the wild type cDNA. One nucleotide exchange was revealed, which was mapped in the region encoding the isomaltase subunit. This mutation, T<sup>3</sup>C at nucleotide 1903, results in a substitution of a cysteine by an arginine at amino acid residue 635 (C635R).

Table 1 compiles the SI primary sequence in different species and demonstrates that the Cys-635 of isomaltase is conserved. This together

FIGURE 2. Processing kinetics of SI and SI<sub>C635R</sub>. A, pulse-chase experiment was performed with wild type SI (WT) and SI<sub>C635R</sub> (Mut). Transiently transfected COS-1 cells were labeled with [<sup>35</sup>S]methionine for 1 h and chased in Dulbecco’s modified Eagle’s medium enriched with 2.5 mM methionine up to 6 h. After lysis, the wild type and mutant proteins were immunoprecipitated with mAb-anti-SI and analyzed on 6% SDS-PAGE and by phosphorimaging. B, the gel in A and two similar ones were scanned and quantified to compare the proportions of the mannose-rich (SI<sub>h</sub>) with those of the mature complex glycosylated forms (SI<sub>c</sub>). The error bars represent S.D.

FIGURE 3. Subcellular localization of mutant SI<sub>C635R</sub> and its wild type counterpart SI. The YFP-fusion forms of wild type SI, SI-YFP, and mutant SI<sub>C635R</sub>, SI<sub>C635R</sub>-YFP, were expressed in COS-1 cells, and their subcellular localization was compared by confocal microscopy. The images show a predominant localization of SI-YFP in the Golgi apparatus and at the plasma membrane while SI<sub>C635R</sub>-YFP shows mostly ER and Golgi staining. A, cotransfection of SI-YFP and SI<sub>C635R</sub>-YFP with the ER-marker dsRed-ER shows a strong localization of the mutant in the ER. B, cotransfection of SI-YFP and SI<sub>C635R</sub>-YFP with the Golgi-marker galactosyltransferase-cyan fluorescent protein (GT-CFP). The localization of SI<sub>C635R</sub>-YFP in the Golgi is markedly reduced as compared with SI-YFP. Scale bars, 20 μm.
with the possible implication of cysteine in a disulfide bond formation suggests that this residue may play a crucial role in the context of a folding determinant. We therefore set out to investigate the role of the residue Cys-635 in sorting and trafficking events of newly synthesized SI by highlighting possible differences between wild type SI and SI bearing the C635R mutation. For this, the mutation C635R was introduced into wild type SI to generate the mutant indicated thereafter SIC635R.

Structural features and intracellular processing of SIC635R were first compared with those of wild type SI in biosynthetic labeling experiments combined with endo H treatment to assess the mannose-rich and complex glycosylated forms. Fig. 1B shows that SIC635R comprised two biosynthetic forms similar to its wild type counterpart: the mannose-rich endo H-sensitive and complex glycosylated endo H-resistant forms. This indicated that the biosynthetic forms of the wild type and the mutant proteins are similar. The relationship of these two biosynthetic forms to each other, however, varied substantially in the mutant protein as compared with wild type SI. Here, mannose-rich SIC635R was the predominant form at this time point and constituted almost 90% of the total synthesized mutant protein. By contrast, wild type SI contained almost equal proportions of Sih (55%) and Sic (45%).

The substantial reduction in the proportion of the complex glycosylated SIC635R is compatible with an effect of the C635R substitution on the conversion rate of the mannose-rich species to the mature protein. We delineated therefore the trafficking kinetics of SIC635R in comparison to the wild type protein in a pulse-chase experiment. Fig. 2 demonstrates that the conversion of the mannose-rich form of SIC635R to the complex form occurs at a slow rate with a half-life of over 6 h (Fig. 2B) with substantial proportions of the mannose-rich form persisting even after 6 h of chase. The wild type SI on the other hand is more rapidly processed as has been previously shown (13), and ~70% is converted into the 245-kDa complex form with increasing chase time points.

Altogether these findings support a direct correlation between the pathologic phenotype of SI and its altered trafficking because of the C635R mutation. Moreover, the reduced rate of complex glycosylation of SIC635R suggests a prolonged intracellular localization of the mutant species.

In light of the biochemical analyses we compared the subcellular and cell surface localization of mutant SIC635R with that of the wild type using chimeras of both proteins that have been fused to the yellow fluorescent protein. Fig. 3 demonstrates a predominant intracellular localization of the mutant protein. Most notably the ER is strongly labeled as assessed by an ER marker (Fig. 3A). Regions of the Golgi apparatus were also colabeled with galactosyltransferase and the mutant, however, to a lesser extent than with the wild type protein (Fig. 3B).

The cell surface localization of SIC635R was also compared with that of the wild type protein employing cell surface immunoprecipitation of biosynthetically labeled cells. At 6 h of chase both proteins acquired complex glycosylation and were isolated from the cell surface (Fig. 4A). However, the proportion of the mutant SIC635R protein at the cell surface as compared with its endogenous counterpart was almost half that of cell surface wild type SI. This suggests that the trafficking rate of complex glycosylated mutant SIC635R from the Golgi to the cell surface differs from that of the wild type protein and raises the question of a possible role of mechanisms that control protein transport in the Golgi. The C635R Alters the Folding of Mutant SIC635R—It is likely that the C635R mutation has induced folding alterations in the mutant SI that are subsequently the underlying cause of these differences in trafficking and processing rates. We therefore followed three approaches to determine whether the C635R substitution has induced gross structural alterations in SI.

In the first approach, epitope mapping of mutant and wild type SI with conformation-specific antibodies was performed. The second utilized probing the folding of the mutant and comparing it with the wild type species by a protease-sensitivity assay using trypsin. Finally, the enzymatic activity of the mutant SI protein was compared with its wild type counterpart.

The previously described antibodies, HBB 1/691, HBB 2/614, HBB 2/219, and HBB 3/705 (14) were used in the epitope mapping. Fig. 5 shows that the antibodies HBB 2/219, HBB 2/614, and HBB 1/691 recognized the mannose-rich and complex forms of the wild type and mutant proteins, albeit with varying affinities. Remarkably, however, the binding capacity of the HBB 3/705 antibody varied substantially between the SI and the wild type protein. This antibody bound avidly the mannose-rich form of SIC635R mutant, whereas it reacted weakly with the wild type protein. It has been demonstrated that this antibody preferentially binds early forms of SI before they have reached the Golgi apparatus. Moreover, it recognizes denatured forms of SI on Western blots (14). The characteristics of this antibody and its strong
binding to the Slc635R mutant are clearly compatible with an altered folding pattern of the mutant as a consequence of the mutation.

The epitope mapping data were further corroborated by a protease-sensitivity assay employing trypsin. In the intestinal lumen pancreatic trypsin cleaves mature SI when it is delivered to the brush-border membrane. The two generated subunits, sucrase and isomaltase, thereafter acquire trypsin resistance. As such trypsin is a quite convenient protease that could be used to probe possible folding alterations, whereby variations in the tryptic digestion pattern are synonymous for a conformational change in the SI protein.

First, trypsin was applied at concentrations that are capable of cleaving wild type SI to the two subunits, which thereafter maintain resistance to further cleavage by the protease. Wild type and mutant SI were isolated from cells that have been labeled for 1 or 4 h to isolate the mannose-rich and complex glycosylated forms of SI and to assess their respective sensitivities toward trypsin. As such trypsin is a quite convenient protease that could be used to probe possible folding alterations, whereby variations in the tryptic digestion pattern are synonymous for a conformational change in the SI protein.

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The observation that the mutant protein acquires complex glycosylation in the Golgi, albeit at reduced rates, suggests that this mutant attains a partial conformational maturation sufficient for its exit from
the ER to the Golgi. We therefore utilized milder digestion conditions to determine whether partial resistance to trypsin can be observed and intermediate folding determinants can be detected. Fig. 6B demonstrates that wild type SI was hardly digested at a lower trypsin concentration. By contrast, multiple digestion products of SLC635R were detected during several biosynthetic intervals comprising the sucrase and isomaltase subunits indicative of maturation of the mutant SI. The mutation C635R therefore did not completely prevent the protein from being correctly folded, but it has affected the maturation kinetics by delaying the acquisition of a native structure.

Finally, the enzymatic activities of the mutant toward the substrates sucrose and isomaltose were analyzed. Here, a substantial decrease in the activity of sucrase was observed, whereas the activity of isomaltase was below detection limit. These measurements are indicative of an altered folding at least around the activity centers of the isomaltase and sucrase subunits (Fig. 7).

The Mutation C635R Increases the Turnover Rate of the Mutant SI—Further we addressed the question whether the observed changes in the folding pattern are associated with an altered turnover rate. For this the turnover of the mutant SLC635R was compared with that of the wild type protein in pulse-chase experiments. It became obvious in these experiments that mutant SI started to disappear steadily throughout the chase time points and was no more detectable after 48 h of chase. By contrast, wild type SI was still persistent, predominantly in its complex glycosylated form at the same time point. This finding is in agreement with the folding analyses, which altogether show that the mutation C635R destabilizes the protein by impairing the folding and promoting a faster turnover (Fig. 8). The more rapid turnover of the SI mutant contributes, therefore, together with the reduced enzymatic activity to the CSID phenotype.

Random Distribution of the Mutant SI in Polarized Cells—Although the mutant protein partially matures in COS-1 cells, acquires activity in its sucrase subunit and is delivered to the cell surface, its possible distribution at the cell surface in polarized cells has still to be assessed. This is important in view of the possibility that missorting could also contribute to the CSID phenotype. We therefore analyzed the sorting of the mutant in MDCK cells that have been transfected with a chimera of the mutant to which the yellow fluorescent protein has been fused. Confocal laser microscopy revealed an almost random distribution of the SLC635R mutant on both the apical and the basolateral cell surfaces. Wild type SI, on the other hand, reproduced the expected distribution profile of the protein on the apical surface of MDCK cells (18) (Fig. 9A). The mutation C635R therefore does not only induce a slower cellular trans-
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Another explanation for the substantial increase in the distribution of the mutant SI<sub>C635R</sub> at the basolateral membrane. Along this, the altered folding of the protein may render it more susceptible to proteolytic cleavage at the apical membrane than at the basolateral membrane. Consequently, the steady state distribution of the mutant SI will shift from an almost exclusive apical location toward a more random distribution at both sides of the membrane concomitant with an overall more rapid turnover. Several protein hydrolases, e.g. endopeptidases, aminopeptidases and peptidases, are largely abundant at the apical, but not the basolateral membrane, of many epithelial cells and may contribute to the cleavage of mutant SI at the apical membrane. Polarized protein sorting in simple epithelial cells, such as in MDCK, Caco-2 or HT-29 cells, is predominantly achieved by selective signal-mediated targeting. The random distribution of mutant SI in this case is likely because of its stabilization at the basolateral surface by, for example, interactions with the submembrane cytoskeleton.

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FIGURE 10. Association of SI and SI<sub>C635R</sub> with detergent-resistant membranes (DRMs). SI and SI<sub>C635R</sub> were expressed in COS-1 cells, which were labeled with [35S]methionine for 6 h. Cells were lysed with 1% Triton X-100 on ice, and the postnuclear supernatant was subjected to ultracentrifugation. The supernatant (S) and pellet (P) were immunoprecipitated with anti-SI mAb and analyzed by SDS-PAGE on 6% slab gels followed by phosphorimaging.

| SI<sub> WT</sub> | SI<sub>C635R</sub> |
|---|---|
| P | S |
| 245 kDa | 210 kDa |

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