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A mutation in a highly conserved region in brush-border sucrase-isomaltase and lysosomal α -glucosidase results in Golgi retention

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SUMMARY

A point mutation in the cDNA of human intestinal sucraseisomaltase has been recently identified in phenotype II of congenital sucrase-isomaltase deficiency. The mutation results in a substitution of glutamine by proline at position 1098 (Q1098P) in the sucrase subunit. Expression of this mutant sucrase-isomaltase cDNA in COS-1 cells results in an accumulation of sucrase-isomaltase in the ER, intermediate compartment and the *cis*-Golgi cisternae similar to the accumulation in phenotype II intestinal cells. An interesting feature of the Q1098P substitution is its location in a region of the sucrase subunit that shares striking similarities with the isomaltase subunit and other functionally related enzymes, such as human lysosomal acid α -glucosidase and Schwanniomyces occidentalis glucoamylase. We speculated that the $Q \rightarrow P$ substitution in these highly conserved regions may result in a comparable accumula-

tion. Here we examined this hypothesis using lysosomal α glucosidase as a reporter gene. Mutagenesis of the glutamine residue at position 244 in the homologous region of α -glucosidase to proline results in a protein that is neither transported to the lysosomes nor secreted extracellularly but accumulates in the ER, intermediate compartment and *cis*-Golgi as a mannose-rich polypeptide similar to mutant sucrase-isomaltase in phenotype II.

We propose that the Q1098P and Q244P mutations (in sucrase-isomaltase and α -glucosidase, respectively) generate structural alterations that are recognized by a control mechanism, operating beyond the ER in the intermediate compartment or *cis*-Golgi.

Key words: ERGIC, Intestine, Trafficking, Quality control, SI deficiency

INTRODUCTION

Transport of proteins along the secretory pathway is a complicated multistep process involving numerous checkpoints that define the ultimate fate of a newly synthesized protein. During recent years our understanding of the molecular events that are implicated in and that control the trafficking of secretory and membrane proteins from one cellular organelle to the other has evolved greatly (Rothman and Orci, 1992; Hong and Tang, 1993; Pelham and Munro, 1993; Rothman and Wieland, 1996). For instance proteins destined for the cell surface or for secretion into the exterior milieu are extensively modified in the ER before their further transport to the Golgi (for reviews) see Pfeffer and Rothman, 1987; Doms et al., 1993). The chemical structure of these proteins as well as their conformation become altered after they have been synthesized and translocated across the ER membrane (Gething et al., 1989). The transport-competent conformation that proteins acquire prior to their transport from the ER to the Golgi and beyond is generally dependent on the attainment of a correct folding and

assembly into a proper quaternary structure (Pelham, 1989). The observed variations in the transport rates of proteins from the ER to the Golgi are presumably linked to different kinetics of protein folding and oligomerization, for example, via chaperon-mediated events (Hurtley and Helenius, 1989; Gething and Sambrook, 1992; Tatu et al., 1995). Consequently, the ER-to-Golgi transport could be considered as being rate limiting along the secretory pathway. Nevertheless, conformational maturation in the Golgi (Jascur et al., 1991; Matter and Hauri, 1991), for instance due to carbohydrate modification (Naim and Lentze, 1992), or oligomerization (Jascur et al., 1991; Lösch et al., 1996), may also affect the transport rates from the Golgi to the cell surface. This suggests that a ratelimiting step also exists at the level of the Golgi and may at least in part be responsible for the asynchronous intracellular transport of brush border enzymes (Hauri et al., 1985; Naim et al., 1988b,c; Stieger et al., 1988; Naim and Lentze, 1992). In general, proteins leaving the ER are subjected to a 'quality' control' mechanism that is responsible for sorting correctly folded from conformationally immature proteins, whereby

misfolded proteins are retained and properly folded molecules leave the ER and traverse the Golgi on the way to their final destinations (Hurtley and Helenius, 1989; Hammond et al., 1994; Ou et al., 1993).

Several observations suggest that not all proteins conform to this general scheme. Firstly, in congenital sucrase-isomaltase deficiency (CSID) two naturally occurring mutants of sucraseisomaltase (SI) reveal folding patterns different from wild-type SI, while both are able to leave the ER (Naim et al., 1988a; Fransen et al., 1991). In one case, the mutant SI undergoes intracellular arrest as an unprocessed mannose-rich precursor in the medial and trans-Golgi (Naim et al., 1988a), and the other phenotype is sorted to the basolateral, rather than the apical membrane (Fransen et al., 1991). Secondly, several chimeric mutants of the hemagglutinin glycoprotein, which persist as monomers in contrast to the trimeric wild-type molecule, reveal different folding patterns, and yet are able to egress the ER, in many cases with wild-type kinetics (Lazarovits and Roth, 1988). Finally, a temperature sensitive mutant of the vesicular stomatitis virus (VSV) glycoprotein (G protein) has been shown to recycle as a malfolded species between the ER, the intermediate compartment and the Golgi (Hammond and Helenius, 1994). These findings raise questions as to whether 'minimal folding requirements' exist, which should be fulfilled by secretory and membrane proteins before egress from the ER is tolerated. Additionally, there could exist a quality control mechanism acting beyond the ER. Studies on the transport of mutant glycoproteins could give insight into these questions. We set out to investigate the molecular characteristics and the fate of a naturally occurring mutant form of sucrase-isomaltase in which the substitution Q1098P causes arrest of SI in the *cis*-Golgi and intermediate compartment. The same $Q \rightarrow P$ mutation was introduced by site-directed mutagenesis into the structurally homologous lysosomal α -glucosidase at position 244 in the evolutionary conserved region. Expression studies demonstrate that the $Q \rightarrow P$ mutation in both proteins has a similar effect. The structural alteration resulting from this mutation seems to be either recognized by a quality control mechanism operating beyond the ER or to have altered the recognition site for the hypothetical 'transport receptors' as described by Rothman and Wieland (1996). Both scenarios result in a retention of the proteins in the intermediate compartment or *cis*-Golgi, where they are finally degraded.

Table 1. Oligonucleotides used in site-directed mutagenesis of lysosomal α-glucosidase

Oligo	bp (Hoefsloot et al., 1988)	Sequence $5' \rightarrow 3'$
Glul	217-234 (coding)	ACCATGGGAGTGAGGCAC
Glu2	960-940 (non-coding; T950G)	CTGAAGGAACGGATCCGCAAA
Glu3	937-957 (coding; A950C)	TTCTTTGCGGA T CCGTTCCTT
Glu4	1239-1222 (non-coding)	GTAGACATCCAGGATCCC

compartment mAb ERGIC-53 was a product of hybridoma G1/93 (Schweizer et al., 1988). Antibodies against human acid α -glucosidase comprised a product of hybridoma 43D1 (Hilkens et al., 1981) and a rabbit polyclonal antiserum which was generously provided by Dr A. J. Reuser (Reuser et al., 1985). Polyclonal anti-protein disulphide isomerase (PDI, Freedman et al., 1989) was kindly provided by Dr N. J. Bulleid, Manchester, UK, and polyclonal anti-SI was kindly provided by Dr A. Zweibaum, INSERM U178, Paris, France.

cDNA probes

Full length wild-type and Q1098P mutant SI cDNAs (Ouwendijk et al., 1996) were subcloned into the mammalian expression vectors pCB7 (derived from pCB6; Brewer and Roth, 1991; Gritz and Davies, 1983) and PSG5 (Green et al., 1988). Wild-type α -glucosidase cDNA was cloned into the pSG5 expression vector as described before (Hoefsloot et al., 1990) to generate pSHAG2.

A mutation A/C at nucleotide 950 (Hoefsloot et al., 1988) of α -glucosidase generates a substitution of glutamine by a proline at amino acid residue 244. This substitution corresponds with the one found at position 1,098 in mutant sucrase-isomaltase.

Mutagenesis of the α -glucosidase cDNA was performed by PCR. Here, the primer Glu2 directed against the non-coding strand was designed to contain the A/C mutation at nucleotide 950 (see Table 1). This primer together with Glu1 in the 5'-3' direction (Table 1) were used to amplify a 743 bp fragment (denoted a-Glu I) (nucleotides 217 to 960). Another pair of primers, Glu3 in the 5'-3' direction and Glu4 for the complementary strand (see Table 1) were used to amplify a 302 bp DNA fragment encompassing nucleotides 937 to 1,239 (denoted a-Glu II). Glu3 contained also the A/C mutation at nucleotide 950. For cloning purposes another mutation was introduced at position 948. This C/T mutation is silent but creates an additional *Bam*HI-site. The DNA fragments a-Glu I and a-Glu II contained 18 overlapping bp between nucleotides 940 and 957. These two fragments were assembled by assembly PCR using the primers Glul and Glu4 to produce a 1,022 bp DNA fragment (nucleotides 217 to 1,239). This PCR product was digested with SacI/Stul to generate a 348 bp fragment. This fragment replaced the Sacl-Stul fragment in the wild-type α -glucosidase cDNA. Sequence analysis of the 348 bp exchanged fragment demonstrated that only the mutations A950C and C948T were present (not shown). Sequencing was performed with a DNA sequencing kit according to the manufacturer's instructions (Sequenase 2.0, United States Biochemical Corporation, Cleveland, OH). No other sequence alterations were found. The mutant α -glucosidase cDNA was subcloned into the pSG5 expression vector to generate the plasmid pSHAG2A/C which was subsequently used in transfection experiments.

MATERIALS AND METHODS

Cell lines

Monkey kidney COS cells (ATCC CRL-I650) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics. Caco-2 cells (ATCC HTB-37) were cultured in DMEM supplemented with 20% heat inactivated FCS, 1% non-essential amino acids and antibiotics (all from Gibco BRL Life Technologies GmbH, Eggenstein, Germany). All cell lines were cultured at 37°C in a humidified 5% CO₂ incubator.

Transfection of COS-1 cells

COS-1 cells were either transfected by the DEAE-dextran method as

Antibodies

Four epitope-specific monoclonal antibodies directed against sucrase, isomaltase or sucrase-isomaltase were used (Hauri et al., 1985). These antibodies were products of the following hybridomas: HBB 1/691, HBB 2/614, HBB 2/219, HBB 3/705. Anti-ER-Golgi intermediate described by Naim et al. (1991a) or via electroporation as described before (Ouwendijk et al., 1996).

Metabolic labeling

Caco-2 cells or transiently transfected COS-1 cells were metabolically labeled with 80 μ Ci L-[³⁵S]methionine (ICN Biomedicals, Meckenheim, Germany) as described by Naim et al. (1991a). After the labeling period, the cells were washed and stored at -70°C until use.

Immunoprecipitation and SDS-PAGE

Metabolically-labeled Caco-2 cells or transfected COS-1 cells were lysed at 4°C for 1 hour in lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxy cholate and a mixture of protease inhibitors containing 1 mM PMSF, 1 µg/ml pepstatin, 5 μ g/ml leupeptin, 80 μ g/ml soybean trypsin inhibitor and j ug/ml aprotinin, all from Sigma, Deisenhofen, Germany). Usually ml ice-cold lysis buffer was used for each 100 mm culture dish (about 2-4×10⁶ cells). The mixture of protease inhibitors was omitted from the lysis buffer when the sensitivity of SI towards trypsin was assessed (see later). Detergent extracts of cells were centrifuged for 1 hour at 100,000 g at 4°C and the supernatants were immunoprecipitated as described by Naim et al. (1991a). For epitope mapping studies with four epitope-specific monoclonal antibodies directed against sucrase, isomaltase or sucrase-isomaltase the extracts of Caco-2 cells or transfected COS-1 cells were divided into equal aliquots and each aliquot was immunoprecipitated with a different antibody. Usually, 0.2μ l mAb in the form of ascites was used for each immunoprecipitation. Human α -glucosidase was immunoprecipitated using 0.5 μ l of a rabbit polyclonal anti-human α -glucosidase serum (Reuser et al., 1985). SDS-PAGE was performed according to the method of Laemmli (1970) and the apparent molecular masses were assessed by comparison with high molecular mass markers (Bio-Rad Laboratories GmbH, München, Germany) run on the same gel. In some experiments, deglycosylation of the immunoprecipitates with Endo-N-acetylglucosaminidase H (Endo H), Endo-N-acetylglucosaminidase F/glycopeptidase F (Endo F/GF, also known as PNGase F) (both from New England Biolabs GmbH, Schwalbach/Taunus, Germany) was performed prior to SDS-PAGE analysis as described before (Naim et al., 1987).

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and spun down in 10% gelatin. Pellets were fixed and stored until use in 1% PFA. Ultrathin cryosectioning was performed as described before (Schweizer et al., 1988; Fransen et al., 1985). Sections were incubated with mAbs against SI (HBB 2/614/88) or α -glucosidase (43D1), followed by a rabbit polyclonal serum against mouse IgG (Dako A/S, Glostrup, Denmark) and Protein A complexed with 10 nm gold (Fransen et al., 1985). Electron microscopy was performed with a JEOL 1010 electron microscope.

RESULTS

Human sucrase-isomaltase expressed in COS-1 cells reveals similar structural and biosynthetic features as the naturally expressed enzyme in intestinal cells

Trypsin treatments

Metabolically labeled cells were solubilized in lysis buffer without protease inhibitors and subsequently treated with trypsin (500 μ g/ml, Sigma, Deisenhofen, Germany) for various time periods or with various trypsin concentrations for 30 minutes at 37°C. The reaction was stopped by cooling on ice and the addition of soybean trypsin inhibitor (Sigma, Deisenhofen, Germany) and protease inhibitors.

Wild-type SI produced in transfected COS-1 cells is indistinguishable in its behavior on SDS-PAGE from the SI naturally produced in intestinal epithelial cells (Ouwendijk et al., 1996). Furthermore, the wild-type enzyme expressed in COS-1 cells was processed with the same rate as in intestinal cells and showed comparable enzymatic activities (not shown) and glycosylation patterns: the 245 kDa complex glycosylated form appears after approximately the same times of chase (Fig. 1). It can therefore be assumed that the folding pattern of the wild-type SI expressed in COS-1 cells is normal.

The enzyme expressed in COS-1 cells was further investigated with respect to trypsin sensitivity, an indicator for protein folding. For this purpose Caco-2 cells and transfected COS-1 cells were biosynthetically labeled for 5 hours and the detergent extracts were treated with trypsin. Under these conditions the mannose-rich SI precursor (pro-SI) as well as the complex glycosylated form become accessible to the protease. Cell lysis without protease inhibitors did not result in proteolytic cleavage of pro-SI (Fig. 2, lane 2 and 7). Likewise both cell types behaved similarly when trypsin was added to the lysates. The pattern obtained with trypsin in both cell types is comparable and was maintained at 500 µg of trypsin for at least 3 hours (Fig. 2, lanes 5 and 11). The observed differences in the intensity of the proteins derived from Caco-2 and transfected COS-1 cells (compare lanes 1-5 with 6-11) are most

Enzyme activities

Disaccharidase activities were measured according to the method of Dahlqvist (1968), using sucrose and isomaltose as substrates. Protein quantities were determined by western blotting and densitometric scanning of fluorograms.

Immunofluorescence

Cellular localization of expressed proteins in COS-1 cells was studied with cells grown on coverslips. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. Immunolabeling was carried out using mAb anti-SI (HBB1/614), mAb anti-ERGIC-53, polyclonal anti-SI, polyclonal anti-PDI, mAb anti- α -glucosidase (43D1) and polyclonal anti-human α -glucosidase as the primary antibodies. The secondary antibodies employed FITCconjugated goat anti-mouse or swine anti-rabbit IgG, Texas Red-conjugated goat anti-mouse or anti-rabbit IgG (all were from Boehringer Mannheim BV, Almere, the Netherlands). Surface localization of proteins was assessed in transfected cells that were not fixed nor permeabilized; labeling was carried out at 4°C. Label was visualized with a Bio-Rad MRC1000 confocal scanning laser microscope using a double channel for fluorescein isothiocyanate and Texas Red or on a routine fluorescence microscope.



Immuno electron microscopy

Ultrastructural localization studies were performed on transfected COS-1 cells. Cells were fixed with 1% paraformaldehyde (PFA) and 0.1% glutaraldehyde in phosphate buffer (pH 7.3) for 1 hour, scraped

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Fig. 1. Processing of SI in COS-1 and Caco-2 cells. Caco-2 and transfected COS-1 cells were biosynthetically labeled with [³⁵S]methionine for 1.5 hours, and chased for the indicated times. Homogenates of these specimens were immunoprecipitated with monoclonal anti-SI antibodies. Immunoprecipitates were subjected to SDS-PAGE on 5% slab gels. Gels were analyzed by fluorography.





Fig. 3. Processing of wild-type (Wt) and mutant (Q1098P) SI in COS-1 cells. Cells were biosynthetically labeled with [³⁵S]methionine for 1.5 hours, and chased for the indicated times. Homogenates of these specimens were immunoprecipitated with monoclonal anti-SI antibodies. Immunoprecipitates were subjected to SDS-PAGE on 5% slab gels. Gels were analyzed by fluorography.

1 2 3 4 5 6 7 8 9 10 11

Fig. 2. Trypsin sensitivity of SI in Caco-2 cells and COS-1 cells. Caco-2 and transfected COS-1 cells were biosynthetically labeled with [35 S]methionine for 5 hours. The specimens were homogenized, solubilized and treated with 500 µg/ml trypsin for the indicated times. After the reaction was stopped with protease inhibitors the samples were immunoprecipitated with monoclonal anti-SI antibodies. The immunoprecipitates were subjected to SDS-PAGE on 5% slab gels. Gels were analyzed by fluorography.

likely due to differences in the labeling efficiency of the two cell types.

Since differently folded molecules are expected to behave differently towards the same protease, in this case trypsin, the results lend strong support to the notion that the folding of pro-SI in Caco-2 cells and COS-1 cells is similar.

To corroborate these data by another approach, we probed

mannose polypeptide during its entire life cycle. The intensity of this form becomes weaker with increasing chase periods indicating that the protein undergoes intracellular degradation, most likely in the ER. Degradation in the lysosomes could be excluded, since the mutant SI protein is not tranported beyond the ER, ERGIC and the *cis*-Golgi (see later Figs 7 and 8).

To investigate whether the mutant enzyme would acquire additional trypsin sensitive sites when expressed in COS-1 cells the experiments described in Fig. 2 were repeated with transfected COS-1 cells expressing wild-type or mutant SI. Fig. 4 shows that neither mutant pro-SI nor the anticipated sucrase or isomaltase subunits were visible after lysis of the cells without protease inhibitors (Fig. 4, 2nd lane) indicating that the mutant pro-SI underwent complete degradation after exposure to endogenous proteases. Wild-type SI was processed correctly within 30 minutes of trypsin treatment (Fig. 4, 'Wt'). The highmannose as well as the complex-glycosylated species of

the folding of pro-SI with epitope-specific monoclonal antibodies. These mAbs recognize specific conformations or different epitopes of SI (Hauri et al., 1985). Therefore, the binding of these antibodies to pro-SI in COS-1 cells would be consistent with the notion that the corresponding epitopes on pro-SI in COS-1 cells and in intestinal cells display comparable folding patterns. All antibodies reacted with pro-SI from both cell types and with comparable affinities (not shown). Since the epitopes recognized by these antibodies are randomly distributed over the pro-SI molecule, it is reasonable to assume that the overall folding pattern of pro-SI in both cell types is comparable or similar.

Altogether, the protease sensitivity measurements and the epitope mappings with four monoclonal antibodies indicate that no gross structural differences exist between pro-SI in COS-I and Caco-2 cells.

Analysis of the folding pattern of mutant human sucrase-isomaltase expressed in COS-1 cells

The mutation Q1098P elicits similar effects on the posttrans-



Fig. 4. Trypsin sensitivity of mutant Q1098P and wild-type (Wt) SI. Transfected COS-1 cells were biosynthetically labeled with [³⁵S]methionine for 5 hours. The specimens were homogenized, solubilized and treated for 30 minutes at 37°C with the indicated trypsin concentrations. After the reaction was stopped with protease inhibitors the samples were immunoprecipitated with monoclonal anti-SI antibodies. The immunoprecipitates were subjected to SDS-PAGE on 5% slab gels. Gels were analyzed by fluorography. In these experiments, mutant SI transfected cells were less efficiently labeled (compare 1st lane 'Q1098P' with 1st lane 'Wt'). Se, Ie, proSIc: complex glycosylated sucrase subunit, isomaltase subunit, proSI (resp.); Sh, Ih, proSIh: high mannose sucrase subunit, isomaltase subunit, proSI (resp.).

lational processing of SI in transfected COS-1 cells as in intestinal epithelial cells (Ouwendijk et al., 1996). In both cell types the mutant protein is not complex glycosylated, but accumulates as a high-mannose precursor in the ER, intermediate compartment and the *cis*-Golgi. We investigated the biosynthesis of mutant SI in transfected COS-1 cells by pulse-chase analysis. As shown in Fig. 3, mutant SI persists as a high-



Fig. 5. Epitope mapping of mutant Q1098P and wild-type (Wt) SI.

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fluorescence using mAb anti-SI HBB 2/614 and FITC-conjugated anti-mouse-IgG on permeabilized or non-permeabilized cells. Bright staining was revealed at the surface of non-permeabilized cells expressing wild-type pro-SI (Fig. 6A). By contrast, mutant pro-SI was not stained at the cell surface and could only be visualized when the cells were permeabilized with Triton X-100 (Fig. 6B).

To characterize more precisely the subcellular localization of mutant SI we used confocal scanning laser microscopy and double labeling with antibodies to SI and to markers for the ER, i.e. PDI (Freedman et al., 1989), or the ER-Golgi intermediate compartment (ERGIC), i.e. ERGIC-53 (Schweizer et al., 1988). Fig. 7 shows that mutant SI colocalizes with PDI (\mathbb{A}) , and with ERGIC-53 (B). These results indicate that mutant SI is not only localized in the ER, but also in the ERGIC. It has been demonstrated that some ERGIC-53 resides also in the cis-Golgi (Hendricks et al., 1991). Wild-type SI showed not only the same labeling patterns as observed with mutant SI, but also an additional, strong cell surface staining could be observed (Fig. 7C and D), indicating that the only difference in localization between wild-type and mutant SI is the cellsurface expression. The exact localization of mutant SI was determined by immuno-electron microscopy. Wild-type SI was localized at the cell surface and in the ER. Non-transfected cells did not show labeling (not shown). Mutant Q1098P SI showed labeling in the ER and in vesicles and membrane structures at one side of the Golgi apparatus (Fig. 8) resembling the ERGIC-structures at the cis-side of the Golgi as described by Schweizer et al. (1988). This pattern exactly matches the pattern observed in the CSID biopsy samples (Ouwendijk et al., 1996). It should be noted that the antibodies used against ERGIC or PDI could not be used for immunoelectron microscopy using COS-1

Transfected COS-1 cells were biosynthetically labeled with [³⁵S]methionine for 4 hours. Solubilized cell homogenates were immunoprecipitated with four different monoclonal anti-SI antibodies (HBB 3/705, HBB 1/691, HBB 2/219, HBB 2/614). Immunoprecipitates were subjected to SDS-PAGE on a 5% slab gel, which was analyzed by fluorography.

sucrase (Sh, Sc) and isomaltase (Ih, Ic) were generated by the trypsin treatment.

The mutant SI was also different from the wild-type enzyme with respect to epitope exposure, and failed to react with mAbs HBB 2/219 and HBB 1/691 (Fig. 5). Together, the proteasesensitivity assays as well as the epitope mapping indicate that the Q1098P mutation has altered the folding of the SI molecule.

Subcellular localization of wild-type and mutant SI expressed in COS-1 cells

The intracellular transport of the wild-type and mutant SI protein in transfected COS-1 cells was examined by immuno-

Fig. 6. Immunofluorescence localization of wild-type SI, mutant Q1098P SI, wildtype α -glucosidase and mutant Q244P α glucosidase in transfected COS-1 cells. All cells except those in A were fixed with paraformaldehyde, permeabilized with Triton



X-100 and labeled with anti-SI mAbs (A,B) or anti- α -glucosidase (C,D) antiserum. (A) Cell surface staining of wild-type SI on non-permeabilized cells. (B) Intracellular labeling of mutant Q1098P SI. (C) Intracellular, lysosomal labeling of wild-type α glucosidase. (D) Intracellular, nonlysosomal labeling of mutant Q244P α glucosidase. Bar, 20 µm.

Fig. 7. Immuno double-labeling of mutant Q1098P SI, wild-type SI and mutant Q244P & glucosidase

with PDI and ERGIC-53 in transfected COS-1 cells. Cells were fixed with paraformaldehyde, permeabilized with Triton X-100 and labeled with anti-SI (A, B, C, D) or anti-o-glucosidase (E,F) antibodies, combined with antibodies against PDI (A, C, E) or against ERGIC-53 (B,D,F). PDI and ERGIC-53 are stained red, SI and α -glucosidase are stained green. When these colors coincide, a yellow staining appears. Mutant Q1098P SI shows colocalization (yellow) with PDI (A) and ERGIC-53 (B). Wild-type SI shows the same colocalizations (C,D) with additional surface staining. Mutant α -glucosidase shows colocalization





with PDI (E) and ERGIC-53 (F). Cells were analyzed on a confocal scanning laser microscope equipped with a Kr/Ar laser. Bar, 25 µm.



Fig. 8. Subcellular localization of mutant SI and mutant α -glucosidase expressed in COS-1 cells. Ultrathin cryosections of fixed transfected COS-1 cells were labeled with monoclonals against SI (2/614/88) or against α -glucosidase (43D1). Mutant Q1098P SI is localized in ER (arrowheads) and in vesicles and tubules at one side of the Golgi apparatus (arrows) resembling ERGIC (Schweizer et al., 1988) and occasionally in some cisternae of the Golgi apparatus (A). Mutant Q244P α -glucosidase shows a comparable localization (B). Bar, 300 nm. cells. However, double labeling was already established by confocal laser scanning microscopy.

Collectively (a) the double-labeling experiments show colocalization with ERGIC-53; (b) the protein is not complex glycosylated, which excludes the possibility that it has reached the *trans*-Golgi; and (c) the EM-localization demonstrate that the Q1098P mutation has induced in a non-epithelial heterologous transfection system a similar phenotype of SI as that identified in the biopsy sample.

Analysis of mutant lysosomal acid α-glucosidase expressed in COS-1 cells

To investigate the influence of the Q1098P mutation in a different protein the Q at position 244 in α -glucosidase was mutated to P. COS-1 cells were transfected with wild-type and

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more, wild-type lysosomal α -glucosidase was partially secreted by COS-1 cells (Fig. 9, lanes 7-9) consistent with previous reports (Hoefsloot et al., 1990). Mutant α -glucosidase, however, was not discerned in the culture medium (Fig. 9, lanes 10-12). Altogether, the results clearly demonstrate that the mutant α -glucosidase has neither been processed in the Golgi apparatus nor secreted into the culture medium.

It is known that lysosomal α -glucosidase undergoes phosphorylation in the cis-Golgi at some of its mannose-residues and is then transported to the lysosomes via binding to the mannose 6-phosphate receptor. On its way to, and in the lysosomes proteolytic processing to a M_r 95,000 intermediate and finally to the M_r 76,000 enzymatically mature protein occurs (Wisselaar et al., 1993; Reuser et al., 1985, 1987). To determine whether mutant α -glucosidase reaches the lysosomes and undergoes cleavage, transfected COS-1 cells were metabolically labeled for 1.5 hours and chased for the indicated time periods (Fig. 10). As a control mock-transfected COS-1 cells underwent the same procedure. Fig. 10 demonstrates that the wild-type protein was intracellularly processed into the M_r 95,000 intermediate and to the M_r 76,000 protein ('cells', upper panel). These two bands were partially sensitive to Endo H treatment, while treatment with Endo F/GF resulted in a further shift of the two species, comparable to the digestion pattern of wild-type α -glucosidase in Fig. 9, lane 1-3 (not shown). The Endo H sensitivity indicates that some mannose-rich chains have not been processed in the Golgi to complex glycosylated glycans. This is mainly due to phosphorylated mannose residues of the carbohydrate chains, which do not undergo further processing in the Golgi apparatus and are required for correct sorting of α -glucosidase to the lysosomes (Wisselaar et al., 1993; Reuser et al., 1985, 1987).

Q244P α -glucosidase cDNA and labeled for 3 hours. The detergent extracts and the culture medium were immunoprecipitated with anti- α -glucosidase antibodies and subjected to SDS-PAGE with or without Endo H and Endo F/GF treatments. As shown in Fig. 9, wild-type α -glucosidase appeared as a polypeptide of M_r 110,000 (lane 1) consistent with previous reports (Wisselaar et al., 1993; Reuser et al., 1985, 1987). Treatment of this polypeptide with Endo H revealed a broad band of M_r 95,000-105,000 (Fig. 9, lane 2). This pattern was not due to incomplete digestion of the mannose-rich chains, since higher concentrations of Endo H did not affect it (not shown). The diffuse pattern of the Endo H product indicates that the M_r 110,000 species comprises Endo H-sensitive as well as Endo H-resistant or partially Endo H-resistant carbohydrate side chains as has been also observed in Caco-2 cells (Klumperman et al., 1991). In fact, Endo F/GF, which cleaves mannose-rich as well as complex type N-linked oligosaccharides, shifted the M_r 110,000 species to a M_r 95,000 band (Fig. 9, lane 3). This result therefore indicates that α -glucosidase arrived in the Golgi, where it acquired complex type of glycans. By contrast, the mutant M_r 110,000 α -glucosidase species persisted completely as a mannose-rich polypeptide, since both Endo H and Endo F/GF generated a similar digestion product of M_r 95,000 (Fig. 9, lanes 4-6). Further-

A proportion of the M_r 110,000 precursor, but neither the M_r 95,000 nor the M_r 76,000 processed forms were secreted by COS-1 cells expressing wild-type α -glucosidase (Fig. 10, 'medium', upper panel). The faint bands in the control ('Cells', lower panel) are a result of a weak reaction of endogenous α -

	Cells					Medium						
Glu	Wt		Q244P		Wt		Q244P		P			
Endo H	-	÷	-	-	+	-	-	+		-	+	-
Endo F	*	-	+	-	**	+	-	-	+	unt	_	+
kDa 110 95	A A A A A A A A A A A A A A A A A											
	1	2	3	4	5	6	7	8	9	10	11	12

Fig. 9. Glycosylation of mutant Q244P and wild-type (Wt) α -



Fig. 10. Processing of mutant Q244P and wild-type (Wt) α -

glucosidase. Transfected COS-1 cells were biosynthetically labeled with [35 S]methionine for 1.5 hours. The cells were homogenized and solubilized. Homogenates and medium were immunoprecipitated with polyclonal anti- α -glucosidase antibodies. The immunoprecipitates were treated with Endo H (lanes 2, 5, 8, 11), Endo F/GF (lanes 3, 6, 9, 12) or left untreated (lanes 1, 4, 7, 10). They were subjected to SDS-PAGE on 7% slab gels. Gels were analyzed by fluorography. glucosidase. COS-1 cells were transfected with cDNA coding for mutant Q244P α -glucosidase, wild-type α -glucosidase (Wt) or mock-transfected (control). The cells were biosynthetically labeled with [³⁵S]methionine for 1.5 hours, chased for the indicated times, homogenized and solubilized. Homogenates and medium were immunoprecipitated with polyclonal anti- α -glucosidase antibodies. Immunoprecipitates were subjected to SDS-PAGE on 7% slab gels. Gels were analyzed by fluorography.

glucosidase with the antibodies. Mutant Q244P α -glucosidase persisted as a high-mannose precursor polypeptide, was not processed to its mature form as the wild-type molecule and was not secreted into the medium (Fig. 10, middle panels). The results indicate that mutant α -glucosidase is neither transported to the cell surface nor to the lysosomes.

Altogether, the results demonstrate that wild-type α -glucosidase is transport-competent since it has been processed in the Golgi, sorted to the lysosomes and partially secreted into the medium (Hoefsloot et al., 1990). By contrast, the mutant α -glucosidase protein persisted as a M_r 110,000, mannose-rich polypeptide and was not processed to the M_r 95,000 or M_r 76,000 forms (Fig. 10, 'cells', middle panel). Furthermore, no secreted M_r 110,000 precursor forms could be detected even after a chase period of 20 hours (Fig. 10, middle panel, 'medium') and longer exposure of the gel (not shown). Together, mutant α -glucosidase is a transport-incompetent molecule that persists as a mannose-rich precursor and is finally degraded, most likely in the ER. these studies the question arose about the possible existence of a quality control mechanism operating at a level beyond the ER.

Here we analyze the Q1098P substitution in more detail and demonstrate that it is not only functional in intestinal epithelial cells but also produces a similar phenotype when expressed in non-polarized COS-1 cells. This supports the view that (i) the Q1098P mutation per se is responsible for the generation of the SI phenotype; and (ii) cellular factors specific for epithelial cells are not implicated in the onset of this CSID phenotype.

The expression of a similar CSID phenotype in COS-1 cells using the patients cDNA enabled us to analyze the processing of the mutant SI protein. One important observation was the folding state of the mutant SI protein. Here protease sensitivity assays using trypsin demonstrate that the mutant SI is not as stable as its wild-type counterpart and is degraded within a relatively short period of time. Interestingly, the sucrase as well as the isomaltase subunits are degraded. While a malfolded sucrase subunit as a result of the mutation Q1098P is expected to exploit a different pattern of trypsin sensitivity as compared to wild-type sucrase, the concomitant degradation of isomaltase can be explained as follows. Wild-type SI does not have its protease resistent conformation directly after translation. It reaches this conformation after a certain 'lag-period' during which both subunits are susceptible to degradation (Matter and Hauri, 1991). Probably mutant SI cannot reach this conformation because: (a) The protein is not transported into the compartment with the proper 'folding machinery'; (b) the mutation makes it energetically impossible to attain the proper folding; or (c) because of the improper folding of sucrase the SI-protein is not transported further, so isomaltase is not folded properly either and remains protease sensitive as well.

Subcellular localization of wild-type and mutant lysosomal α-glucosidase expressed in COS-1 cells

Having assessed the biosynthetic features of mutant α -glucosidase we wanted to determine its subcellular localization and see whether the mutation Q244P has also induced a similar transport block as that observed with mutant SI. Fig. 6C shows a punctate staining pattern typical of the lysosomes as well as ER and Golgi staining in COS-1 cells expressing wild-type α glucosidase. The punctate pattern was completely absent in cells expressing mutant α -glucosidase, and instead immunofluorescence images corresponding predominantly to ER structures were observed (D).

Double-labeling experiments of mutant α -glucosidase with PDI and ERGIC-53 (Fig. 7E and F) resulted in co-localization of mutant α -glucosidase with PDI (E), and ERGIC-53 (F) in regions around the nucleus. Immuno EM analysis of wild-type α -glucosidase expressed in COS-1 cells revealed labeling in the ER, in the lysosomes and at the cell surface (Hoefsloot et al., 1990). Non-transfected cells were devoid of label (not shown). Mutant Q244P α -glucosidase, however, did not show label in the lysosomes nor at the cell surface (not shown), but was localized in the ER and in vesicles and cisternae at one side of the Golgi apparatus (Fig. 8B), comparable to mutant Q1098P SI (Fig. 8A). The results indicate that the mutation Q244P in α -glucosidase has produced a similar phenotype as Q1098P in SI, which is characterized by a block in the transport at the level of ERGIC and *cis*-Golgi. It is therefore concluded that this highly conserved region of the molecules is important for the correct processing and transport of both enzymes.

Alternatively, associations between the sucrase and isomaltase subunits could mask putative trypsin cleavage sites in either subunit preventing further cleavage. One type of association could take place after the independent folding of each subunit. These are ultimately assembled as pseudodimers (Jascur et al., 1991) which efficiently leave the ER. Another type of association could take place very early in the ER, where the subunits possibly assist each other to fold properly and in this way function as 'intramolecular chaperons' (Oberholzer et al., 1993; Sagherian et al., 1994; Naim et al., 1994). In mutant SI, these protective associations might be absent, resulting in exposed protease sensitive sites. Another interesting feature of the Q1098P mutation is its presence in a region that shares striking homologies between human, rat and rabbit sucrase and isomaltase variants as well as human lysosomal α -glucosidase and Schw. occidentalis glucoamylase (Fig. 11) (Naim et al., 1991b). These proteins, which have been suggested to have evolved from a common ancestral gene, are all synthesized and translocated into the ER where they are processed to transport-competent forms and

DISCUSSION

then transported along the secretory pathway to their final destinations. SI is sorted, most likely in the TGN, to the apical membrane. Lysosomal α -glucosidase is after phosphorylation in the *cis*-Golgi and maturation in the medial and *trans*-Golgi mainly transported to the lysosomes, but is also to a lesser extent secreted at the apical membrane in polarized epithelial cells. *Schw. occidentalis* glucoamylase is transported to the

Analysis of congenital sucrase-isomaltase deficiencies has resulted in the identification of different phenotypes showing transport incompetence of the SI protein. In a recent paper we analyzed the cDNA of a phenotype II patient and found a point mutation which generates a Q1098P substitution responsible for a block of the molecule in the ER-Golgi intermediate compartment and the *cis*-Golgi (Ouwendijk et al., 1996). From

	1088	1098	1108	1118
human sucrase	SWLPGF	AFNDQFIQIS	TRLPSEYIY	FGEVEHT
mutant sucrase	SWLPGF	'AFNDPFIQIS	TRLPSEYIY	FGEVEHT
human isomaltase	TSIGPL	VYSDQYLQIS	ARLPSDYIY	SIGEQVHK
human α-glucosidase	TTVAPL	FFADQFLQLS	TSLPSQYITC	LAEHLSP
mutant <i>a-glucosidase</i>	TTVAPL	FFADPFLQLS	TSLPSQYITC	LAEHLSP
	1 234	1 244	254	264

Fig. 11. Partial alignment of the amino acid sequences of human sucrase (Chantret et al., 1992), mutant sucrase (Ouwendijk et al., 1996), human isomaltase (Chantret et al., 1992), human α glucosidase (Hoefsloot et al., 1988) and mutant α -glucosidase. The box indicates a homologous region around the Q1098P mutation. The numbering above the sequences is the sucrase amino acid numbering, below the sequences is the α -glucosidase numbering. Both mutations are typed in boldface.

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1994). In view of these findings the hypothesis has emerged that misfolded proteins that have escaped the ER are retrieved from ERGIC or cis-Golgi back to the ER presumably to be reexposed to the folding machinery and thus have additional opportunities to acquire correct folding. A similar recycling mechanism between the ER and ERGIC or cis-Golgi is potentially possible for mutant SI and lysosomal α -glucosidase. In the case of the VSV G protein a retrieval mechanism to the ER has been proposed that implicates the binding of the BiP/VSV G complex to the BiP receptor in the ER. Although we do not know whether SI or lysosomal α -glucosidase bind BiP or calnexin, a retrieval mechanism of mutant SI or lysosomal α glucosidase complexes with ER-resident proteins cannot be excluded.

The third possibility is that a quality control mechanism operates at a level beyond the ER and prevents misfolded proteins from being further transported along the secretory pathway to the cell surface. After longer periods the mutant proteins are finally degraded. We suggest therefore that the Q1098P mutation in SI and the Q244P mutation in α -glucosidase have generated structural alterations in the proteins that cause an arrest by a control mechanism operating in the intermediate compartment or cis-Golgi. A comparable situation has been reported for misfolded major histocompatibility complex (MHC) class I molecules in mice that are deficient for TAP1, the transporter associated with antigen presentation (Raposo et al., 1995). In these mice the MHC molecules cannot bind to peptides, which prevent the complexes from folding properly. As a result they accumulate in an expanded ER-Golgi intermediate compartment. Although we did not find exactly the same expansion, we did report an enlargement of the Golgi complex and a change in the localization of ERGIC-53 in the patient's intestinal cells (Ouwendijk et al., 1996). In essence, the analysis of CSID has resulted in the identification of a mutation that is responsible for a block of the molecule in the ERGIC or the *cis*-Golgi. Although our results favor a possible quality control mechanism in these compartments, analysis of the binding kinetics of mutant SI and lysosomal α -glucosidase with BiP or calnexin is required to determine whether a recycling mechanism is possible for these proteins. The observations regarding the role of a highly conserved region of SI in proper transport of the molecule could be an interesting contribution to the identification of novel control steps involved in proper transport along the secretory pathway.

periplasmic space and partially secreted into the exterior milieu in yeast. Obviously all three proteins share a common pathway from the ER to the Golgi and from there diverge to different destinations. It is therefore conceivable that homologies in the polypeptide sequences should be implicated in common functions needed for proper transport or catalytic activity. If the mutation Q1098P does not induce gross conformational changes in the sucrase subunit, but rather alters a subdomain that is critical for the intracellular transport of SI, then a similar effect might be expected if the same mutation is introduced into the corresponding homologous regions of lysosomal α -glucosidase or Schw. occidentalis glucoamylase.

Our results show indeed that the Q1098P mutation of sucrase elicits a similar effect when introduced at the corresponding amino acid position 244 in lysosomal α -glucosidase. The mutated lysosomal α -glucosidase precursor as well as the mutant SI precursor remain mannose-rich polypeptides that do not undergo maturation in the Golgi, are not cleaved into mature enzyme and are not transported through the Golgi cisternae. It is conceivable that the homologous domain of α glucosidase, sucrase and isomaltase harbor a structural motive with a potential role in transport signaling or quality control acting at the level of ERGIC-cis-Golgi at which level the mutant proteins are blocked. The observations described here can be explained as follows: (1) the mutation has introduced a retention signal for the *cis*-Golgi; (2) the mutation has lead to the recognition of the protein by the ER-Golgi recycling machinery; or (3) the mutation has lead to a structural alteration that functions as a recognition site for a quality control machinery operating in the intermediate compartment or *cis*-Golgi.

The first possibility suggests that a single point mutation alters a highly conserved region into a region with a novel function. Although it cannot be excluded, we think this is very unlikely.

The second possibility is that mutant SI and lysosomal α glucosidase exit the ER, are transported to ERGIC or *cis*-Golgi and then are recycled back to the ER. Recent observations with

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a temperature sensitive mutant of the VSV G protein have shown that at the non permissive temperature the G protein leaves the ER, arrives in the ERGIC and the cis-Golgi and recycles back to the ER. Presumably several cycles of ER-ERGIC-ER occur during which the G protein is always found associated with the luminal binding protein (BiP) and calnexin in the ER and with BiP in ERGIC (Hammond and Helenius,

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