Biosynthesis of the Human Sucrase-Isomaltase Complex

DIFFERENTIAL 0-GLYCOSYLATION OF THE SUCRASE SUBUNIT CORRELATES WITH ITS POSITION WITHIN THE ENZYME COMPLEX*

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Hassan Y. Naim, Erwin E. Sterchi, and Michael J. Lentze
From the Department of Gastroenterology, Children’s Hospital of the University of Bern, Freiburgstrasse 15, CH-3010 Bern, Switzerland

The biosynthesis and maturation of human sucrase-isomaltase (SI, EC 3.2.1.48-10), was studied in cultured small intestinal biopsy specimens and mucosa explants. Pulse-chase experiments with [35S]methionine revealed one high mannose intermediate of \( M_r = 210,000 \) (pro-SI) which was processed at a slow rate to an endo H-resistant, mature form of \( M_r = 245,000 \) (pro-SI). The fully core-glycosylated form \( (M_r = 212,000) \) was detected only when 1-deoxynojirimycin was added to the culture medium, thus indicating that the core sugars undergo rapid processing by rough endoplasmic reticulum membrane-bound glycosidases. The data presented showed that trypsin specifically and instantaneously (within 1 min) cleaves pro-SI, to two subunits \( L_1 (M_r = 145,000) \) and \( S (M_r = 130,000) \). Elastase and chymotrypsin are not effective.

Enzymic and chemical deglycosylations of SI with endo-\( \beta \)-N-acetylglucosaminidase \( F \)glycopeptidase \( F \) and trifluoromethanesulfonic acid (TFMS) as well as probing for the binding capacity of SI to \( Helix pomatia \) lectin demonstrated that pro-SI, \( L_1 \), and \( S \), are N- and O-glycosylated. Furthermore, the results were indicative of a posttranslational O-glycosylation of pro-SI, since (i) the earliest detectable precursor form, pro-SI, did not bind to \( H. pomatia \) lectin and (ii) its deglycosylation products with both endo-\( \beta \)-N-acetylglucosaminidase \( H \) and TFMS were identical.

Both the \( S \) and \( L_1 \) subunits contain eight N-linked glycan units, at least one of which is of the high mannose type and found on \( S \). Finally, \( S \), but not \( L_1 \), was shown to display at least four populations varying in their content of O-linked glycans. The heterogeneous O-glycosylation pattern of \( S \) could be correlated with the distal position of this subunit (and its O-glycosylation sites) within the pro-SI molecule, thus affecting the extent of O-linked oligosaccharide processing and their subsequent presentation on the mature molecule.

Much of our knowledge of the biosynthesis of plasma membrane proteins has evolved from studies in cultured epithelial cells, such as the Madin-Darby canine kidney cell infected with enveloped viruses (for a review see Ref. 1). These studies revealed the existence of highly organized pathways for viral glycoproteins to enter the host cell, follow distinct sequential routes through the cellular compartments, and segregate in a polarized fashion to the basolateral and apical domains. To which extent the viral model applies to endogenous membrane proteins remains, however, to be determined. This is mainly because cytopathic effects that accompany infections might influence the polarized surface expression of viral glycoproteins.

The epithelial cells of the human small intestine (enterocytes) provide a good experimental model for studies aimed at elucidating the molecular events involved in the biogenesis and trafficking of membrane proteins. These cells are characterized by a striking polarization of their plasma membrane in two distinct structural and functional domains: the brush border or microvillar membrane and the basolateral membrane. The microvillar membrane is endowed with a number of glycoproteins which are involved in the terminal steps of digestion of micromolecular nutrients, and most of them have been characterized (2-4). Sucrase-isomaltase (SI, EC 3.2.1.48-10), an enzyme complex responsible for the final steps in starch and glycogen digestion, is a particularly attractive microvillar membrane glycoprotein to study, as it displays several interesting structural features (5-13). SI is a heterodimeric molecule comprising two subunits of unequal size, sucrase and isomaltase. It is anchored to the brush border (microvillar) membrane by a hydrophobic NH2-terminal segment of the isomaltase subunit. The sucrase subunit lacks a membrane anchor and is associated with the isomaltase subunit on the luminal side of the brush border membrane by noncovalent, strong ionic interactions. Recent structural data for rabbit SI deduced from sequenced cDNA revealed striking homologies between both subunits, so that the consensus is now emerging that the enzyme complex has evolved by partial gene duplication (14). Furthermore, these results provided additional details about the membrane anchoring of SI. Thus it became apparent that SI spans the membrane once, having the amino terminus exposed on the cytoplasmic side, and, conclusively, SI is synthesized without a cleavable signal sequence in a fashion similar to the invariant chain of class II histocompatibility antigens (15-18), the transferrin receptor (19), the asialoglycoprotein receptor (20-22), and the neuraminidase of influenza virus (23). Several studies have shown that sucrase-isomaltase is synthesized as a single chain precursor protein (denoted pro-SI) (7, 24), which is cleaved, once it reaches the microvillar membrane, by pancreatic se-
creations to its two enzymatically active subunits. The protease responsible for this event in the rat small intestine has been shown to be elastase (7). However, this finding was not extended to or confirmed in other species.

Despite extensive work on the structure and topology of SI in many species, several aspects of the intracellular processing have not been explored in great detail. In particular, thorough analyses of the events occurring at the rough endoplasmic reticulum (RER) and the Golgi apparatus are as yet not clearly assessed. For instance, it is not clear whether SI, as is generally believed for other membrane glycoproteins in many cellular systems, undergoes early modification of the carbohydrate residues in the RER (25) and whether this type of modification is crucial for the transport kinetics of the molecule from the RER to the Golgi. Furthermore, although data were presented which were suggestive of an O-glycosylation of SI (26, 27), unequivocal determination of the type (O-linked/N-linked) and the size of the carbohydrate chains on the mature precursor molecule and on both subunits, as well as the correlation of the glycosylation events to the positioning of the subunits within the large precursor molecule, are still lacking. In addition, provided that it takes place, the question as to whether the attachment of O-linked oligosaccharides to the polypeptide backbone is a cotranslational or a posttranslational event has not yet been answered.

Finally, the immense body of data on the structure of SI and its implication for the biosynthesis of the molecule has not been extended to or investigated in great detail in the human species in normal small intestinal epithelial cells (28). Results from such investigations should provide a better knowledge of the overall importance of the expression of sucrase-isomaltase. This is particularly crucial in studies aimed at elucidating the molecular mechanisms underlying the synthesis and processing of sucrase-isomaltase in a number of developmental and pathological situations such as the transient expression of sucrase-isomaltase in the human fetal colon (29), its expression in some human colon cancers (30), or its impaired expression in congenital sucrase-isomaltase deficiency (28, 31). Most interesting cases provide good experimental tools for dissecting the sequence of events during synthesis and intracellular transport of proteins in general at the molecular level.

In this paper, we investigated the biosynthesis of SI in organ culture of human small intestinal epithelial cells, identified precursor and mature forms of SI, analyzed in detail the posttranslational modifications at the RER and in the Golgi, and assessed the type and size of the carbohydrate moieties of both subunits and of the precursor molecules. We show that SI is synthesized as a single polypeptide high mannose precursor that has undergone rapid trimming in the RER, slowly transported to the Golgi where complex glycosylation takes place, and finally is cleaved by trypsin after the attachment of 0-linked oligosaccharides to the polypeptide backbone of the mature precursor molecule and on both subunits, as well as the correlation of the glycosylation events to the positioning of the subunits within the large precursor molecule, are still lacking. In addition, provided that it takes place, the question as to whether the attachment of O-linked oligosaccharides to the polypeptide backbone is a cotranslational or a posttranslational event has not yet been answered.

**EXPERIMENTAL PROCEDURES**

**Materials**

[35S]Methionine (>1000 Ci/mol), Enlightning, and endo-β-N-acetylglucosaminidase H were purchased from Du Pont-New England Nuclear. Acrylamide, N,N'-methylenebisacrylamide, sodium dodecyl sulfate (SDS), Tris, TEMED, ammonium persulfate, 2-mercaptoethanol, dithiothreitol, and Triton X-100 were obtained from Bio-Rad. Tunicamycin, pepstatin, leupeptin, benzamidine, aprotonin, cycloheximide, trypsin (Catalog No. T-2395), elastase (Catalog No. E-0127), soybean trypsin inhibitor, and molecular weight standards for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma. Endo-β-N-acetylglucosaminidase F containing glycopeptidase F, Catalog No. 878 740) and phenylmethanesulfonyl fluoride (PMSF) were obtained from Boehringer Mannheim. Organ tissue culture dishes 60 × 15-mm with center wells were obtained from Falcon, division of Becton-Dickinson and Co. RPMI 1640, methionine-deficient RPMI 1640, streptomycin, penicillin, and fetal calf serum were purchased from Amimed AG, Basel, 1-deoxyo-nigromycin (dNIM) was kindly provided by Dr. Truschiet, Bayer AG, Wuppertal, West Germany. Protein A-Sepharose, cyogent bromide (CNBr)-activated Sepharose, Helix pomatia lectin-Sepharose, and lentil lectin-Sepharose were purchased from Pharmacia Fine Chemicals. Sodium deoxycholate, α-chymotrypsin (Catalog No. 27270), trifluoromethanesulfonic acid (TFMS), anisole, pyridine for ultraviolet spectroscopy, N-acetyl-D-galactosamine, and methyl-α-D-nanopyranoside were obtained from Fluka AG, Switzerland. All other reagents were of superior analytical grade.

**Biological Materials**

Human small intestinal biopsy specimens (approximately 5–10 mg wet weight) were obtained for routine diagnostic purposes by suction with a pediatric Watson capsule. They appeared normal when examined and light microscopy and expressed normal carbohydrate activities (sucrase, 28–80 IU/g, and lactase, 16–49 IU/g, determined according to Asp et al. (32)). Another source for the tissue organ culture material was the small intestine of kidney donors taken directly after the respiratory support system has been switched off. The tissue was transferred to the laboratory within minutes where it was rapidly rinsed with 0.9% cold saline. Intestinal explants were prepared by dissection of the mucosa from the proximal jejunum. They were approximately 2 × 5-mm in size and had normal morphology and disaccharidase activities. The usage of tissue from both sources was in accordance with the rules of the ethical committee of the hospitals of the university.

**Immunological Reagents**

Monoclonal antibodies against the human small intestinal brush border membrane hydrolases were produced according to established hybridoma techniques and described in detail elsewhere (33). The mouse anti-sucrase-isomaltase (anti-SI) monoclonal antibody was a product of hybridoma HBB 2/219/88 and used in ascites form prepared from hybridoma-bearing Pristane-primed Balb/c mice. For immunoprecipitation, anti-SI monoclonal antibody was partially purified from the ascites fluid by two successive precipitations with 45% ammonium sulfate. The precipitates were dissolved in 0.1 M sodium bicarbonate (pH 8.3) containing 0.5 M sodium chloride, extensively dialyzed against the same buffer, and conjugated to CNBr-activated Sepharose 4B according to the manufacturer's instructions. About 4–6 mg of the immunoglobulin fraction was usually coupled to 1 ml of Sepharose.

**Methods**

**Biosynthetic Labeling of Biopsy Specimens (Continuous Pulse)**—Biopsy specimens were washed three times with RPMI 1640 medium supplemented with streptomyein (100 μg/ml), penicillin (100 units/ml), and 10% dialyzed fetal calf serum (designated complete medium) and placed on stainless steel grids in tissue culture dishes essentially as described by Browning and Trier (34). They were then incubated in methionine-deficient RPMI 1640 medium containing 10% fetal calf serum and antibiotics as above (denoted Met-free medium) at 1 ml/biopsy specimen for 2 h at 37 °C in a CO2 + O2 (5:95, v/v) incubator prior to pulse labeling with 150 μCi of [35S]methionine. Continuous labeling was performed for 15 min, 4 h, and 18 h. When used, tunicamycin (5 μg/ml) and dNIM (5 μM) were present during the preincubations of Met-free medium and during pulse labeling. After the labeling periods, the biopsy specimens were chilled to 4 °C, washed three times with ice-cold phosphate-buffered saline, and homogenized with a Teflon-glass homogenizer in 1 ml of 20 mM Tris-HCl (pH 7.4) 50 mM NaCl, and a mixture of protease inhibitors containing 1 mM PMSF, 1 μg of pepstatin, 5 μg of leupeptin, 17.4 μg of benzamidine, and 1 μg of aprotonin (homogenization buffer). The homogenates were either further directly processed for immunoprecipitation or kept (CONT) at −20 °C until used. For studies aimed at examining the effect of pancreatic proteases on cleavage of the polypeptide precursor of sucrase-isomaltase, the protease inhibitor mixture was omitted from the homogenization buffer.
Treatment of Biopsy Homogenates with Pancreatic Proteases—Biopsy specimens were continuously labeled with [35S]methionine for 4 h, homogenized in 4 ml of the homogenization buffer, divided into 4 aliquots, and the effect of three pancreatic proteases was investigated by modifying the procedure of Hauri et al. (7). The aliquots were added to 10 ml of 1 mM bicine buffer (pH 8.2), 0.5 mM CaCl2, 0.5 mM chymotrypsin, or 60 units/ml porcine elastase. The treated aliquots and an untreated control were incubated for 15 min at 37°C. The enzyme reaction was stopped by adding soybean trypsin inhibitor at 1 mg/ml in the case of trypsin or PMSF at 10 mM in the case of α-chymotrypsin or elastase. The reaction mixture was rapidly chilled to 4°C and stored frozen (−20°C) or directly processed for immunoprecipitation. The data obtained from this experiment have shown that trypsin, but not elastase or α-chymotrypsin, was effective in generating sucrase and isomaltase from the precursor molecule pro-sucrase-isomaltase (see "Results," below). The kinetics of this cleavage were examined as follows. Biopsy specimens labeled with [35S]methionine for 4 h were homogenized and divided into several aliquots each containing 1 ml of homogenate equivalent to one biopsy specimen. The aliquots were prewarmed to 37°C and trypsin was added to a final concentration of 0.5 mg/ml. Incubations were carried out for 1, 2, and 5 min at 37°C. Thereafter, the aliquots were further processed as described above. The control sample (zero time) constituted an aliquot to which trypsin and soybean trypsin inhibitor were added and rapidly successively added. The reaction mixture in this case was immediately cooled to 4°C by brief cooling in a slurry of Dry Ice in ethanol.

Biosynthetic Labeling of Mucosa Explants (Pulse-Chase)—Mucosa explants were prepared for biosynthetic labeling as described above for biopsy specimens. Following a pulse period of 10 min with 200 μCi of [35S]methionine, the explants were rapidly washed with ice-cold complete medium and incubated for 2 min at room temperature in the same medium containing 0.1 mg/ml cycloheximide. Samples were chased for various times with complete medium supplemented with 2.5 mM methionine. At each time point an explant was washed in ice-cold phosphate-buffered saline, suspended in the homogenization buffer as described for biopsy specimens, and prepared for immunoprecipitation. When used, monesin (1 μM) was present during the preincubations of Met-free medium, pulse, labeling, and chase.

Preparation of Brush Border Membrane Vesicles (BBM) from Small Intestinal Mucosa—All procedures were carried out at 4°C unless otherwise stated. Mucosa was scraped off with the back of a scalpel blade from a 30-cm piece of frozen and thawed human small intestine, otherwise stated. Mucosa was scraped off with the back of a scalpel chase.

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Elution of Proteins from Fixed and Dried Polyacrylamide Gels and H. pomatia Lectin Chromatography—Proteins from purified brush border membrane fractions (BBM) were identified by Coomassie Blue staining of polyacrylamide slab gels, excised, and eluted essentially as described by Luscher et al. (40) with slight modifications. Briefly, the proteins bands (approximately 7–10 μg each) were swollen in water, transferred to Eppendorf tubes, and incubated overnight at 56°C. The eluted proteins were precipitated with trichloroacetic acid to a final concentration of 15% (w/v). These pellets were further washed and homogenized in phosphate-buffered saline containing 1% Triton X-100, followed by precipitation with trichloroacetic acid and washing on ice-cold acetone and kept at −20°C. Dose-dependent deglycosylation of SI with endo F/GF was carried out as follows. Mucosa explants were biosynthetically labeled for 4 h with [35S]methionine, homogenized, and centrifuged at 10,000 × g for 15 min. The membranes contained in the supernatant were precipitated for 3 min with 0.1% Triton X-100, so that the pro-SI molecules in the outside-in oriented vesicles could be made accessible to trypsin. Subsequently, trypsin was added at 0.5 mg/ml, and the reaction was allowed to proceed for 5 min at 37°C. The homogenates were further homogenized with Triton X-100 and deoxycholate, and detergent extractions and the detergent extracts were divided into equal parts corresponding to one mucosa explant. SI was immunoprecipitated and the precipitates were subjected to endo F/GF treatment with varying concentrations of the endoglycosidase (2–100 milliunits). 

Lectin-Sepharose Affinity Chromatographies—Biopsy specimens labeled for 4 h with [35S]methionine, or nonlabeled brush border membrane fractions (BBM, 2 mg/ml) were homogenized and solubilized in buffer A supplemented with the protease inhibitor mixture (see above) and centrifuged at 100,000 × g for 45 min at 4°C. The supernatant (1 ml) was divided into two equal parts. The supernatant or the pellets were solubilized in phosphate-buffered saline containing 1% Triton X-100 (buffer A, 1 ml/pellet) or additionally treated with endo F/GF followed by precipitation with trichloroacetic acid and washing of the protein pellets with ice-cold acetone. The digestion products were finally separated by precipitation with trichloroacetic acid and washing of the protein pellets with ice-cold acetone. The digestion products were finally separated by precipitation with trichloroacetic acid and washing of the protein pellets with ice-cold acetone. The digestion products were finally separated by precipitation with trichloroacetic acid and washing of the protein pellets with ice-cold acetone.
Deglycosylation of Sucrase-Isomaltase by TFMS—\(^{35}S\)-Labeled molecules were subjected to TFMS according to Edge et al. (41). Due to the low amount of protein in the sample, reproducible results were obtained only when a glycosylated carrier protein was added to the reaction mixture. Sucrase-isomaltase was immunoprecipitated with anti-SI-Sepharose beads, and the beads were eluted in 4% SDS in water by boiling for 5 min. To the eluates, 10 μg of ovalbumin was added, and the mixture was precipitated in 3 volumes of ice-cold acetone for at least 6 h at -20 °C. After centrifugation, the protein pellet was dried at 37 °C and cooled on ice. To the pellet, 30 μl of a precooled mixture of TFMS and anisole (2:1, v/v) were added. The vial was capped after bubbling N\(_2\) through the solution and left for 2.5 h at 0 °C. The reaction was terminated by the addition of 125 μl of pyridine/water (4:1, v/v) in 10-μl portions. During this treatment the vial was immersed in a slurry of acetone/Dry Ice. The solution was precipitated with 3 volumes of ice-cold acetone and washed twice with acetone. A similar treatment was carried out on \(^{35}S\)-labeled molecules which were digested with endo F/GF. Proteins were further analyzed by SDS-PAGE.

RESULTS

Identification of the Precursor and Mature Forms of Human Intestinal SI

To identify and to assess the size of the precursor and mature forms of the SI molecule, biopsy specimens were labeled for 15 min and 4 h with \(^{35}S\)methionine, and SI was immunoprecipitated with monoclonal antibodies. Portions of each sample were treated with endo H, which cleaves glycan units of the high mannose type (42), or endo F (containing glycopeptidase F), which cleaves both the high mannose and complex type of carbohydrates (43). Treated and untreated samples were further analyzed on SDS gels.

Fig. 1A, lane a, shows SI precipitated from biopsies labeled for 15 min. A single polypeptide of \(M_r = 210,000\) was detected. This polypeptide was susceptible to endo H digestion, since treatment caused a substantial decrease in its apparent molecular weight to 185,000 (Fig. 1A, lane b). A similar molecular species was revealed upon endo F/GF treatment (Fig. 1A, lane c). Addition of tunicamycin, which inhibits the co-translational N-glycosylation of proteins (44), to the culture medium before and during labeling of biopsy specimens resulted in the identification of the 185,000 protein by anti-SI antibodies (Fig. 1B, lane b). Taken together, these results indicate that the \(M_r = 210,000\) protein has \(N\)-linked oligosaccharides exclusively of the high mannose type and represents therefore the cotranslationally glycosylated high mannose precursor of SI. Fig. 1A, lane d, shows SI immunoprecipitated from biopsy samples labeled for 4 h. In addition to the \(M_r = 210,000\) protein, a polypeptide of \(M_r = 245,000\) was revealed. The electrophoretic mobility of this species was slightly changed to approximately \(M_r = 243,000\) upon endo H digestion (Fig. 1A, lane e, indicated by the open triangle). In contrast, endo F/GF treatment resulted in a significant shift to \(M_r = 205,000\) and \(M_r = 185,000\) (Fig. 1A, lane f) thus indicating that a large proportion of the \(N\)-linked high mannose oligosaccharides has been processed to complex type of glycans.

To identify the molecular species of SI in the BBM and IM, biopsies metabolically labeled with \(^{35}S\)methionine for 4 h were homogenized, and cellular fractionation was performed by the Ca\(^{2+}\) precipitation method. Both fractions were solubilized and immunoprecipitated with anti-SI antibodies. Fig. 2 shows that the \(M_r = 245,000\) protein was the only component of SI detected in BBM (lane b). The IM fraction contained both the high mannose precursor, i.e. the \(M_r = 210,000\) as well as the \(M_r = 245,000\) proteins (Fig. 2, lane a). The \(M_r = 245,000\) protein is therefore the mature, complex-glycosylated, brush border form of SI revealed in biopsy specimen biosynthetically labeled in organ culture. In brush border membrane preparations from nonlabeled mucosa, SI is revealed as three polypeptides of \(M_r = 245,000, 145,000,\) and 130,000 (Fig. 2, lane c). In analogy with other systems (4, 7–9), these polypeptides correspond to the brush border forms of pro-SI and the subunits isomaltase and sucrase, respectively. That the latter two polypeptides were not detected in biosynthetically labeled biopsies is consistent with the concept of the extracellular cleavage by intraluminal proteases of pro-SI after maturation and insertion into the membrane. However, a considerable proportion of the enzyme persists in the noncleaved precursor form. Densitometric scans of Coomassie Blue-stained gels of electrophoretically analyzed SI showed that the \(M_r = 245,000\) amounts to approximately 20% of the total protein immunoprecipitated from nonlabeled tissue (mean of five preparations, lane c in Fig. 2 is representative for these purifications).
the cell surface by correlating these to the detection of the transport kinetics from the Golgi apparatus (7, 9). The protease responsible for this process in man is not known. Hauri et al. (7) have shown, in the rat small intestine, that pro-SI is cleaved by elastase. In addition, the time course of appearance of the two poly-Glycosylations were identified when higher endo F/GF concentrations were used (not shown). SI purified from the intracellular membrane fraction revealed both pro-SI, and pro-SI\(\text{h}\) (lane c). Endo F/GF digestion of these bands generated the 205,000 species as well as other species (lanes c and d).

The reaction was stopped as indicated under “Experimental Procedures” and further processed for immunoprecipitation with anti-SI-antibodies. The immunoprecipitates were analyzed by SDS-gel electrophoresis and fluorography. B, four biopsy specimens were biosynthetically labeled for 4 h with \(^{[35]}\)S-methionine, homogenized in 4 ml, and divided into four samples each containing 1 ml equivalent to one biopsy sample. The aliquots were prewarmed to 37 °C, and trypsin was added at 0.5 mg/ml final concentration. Incubations were performed for 1, 2, and 5 min. SI was immunoprecipitated and subjected to SDS-gel electrophoresis and fluorography. The fluorograms were evaluated by densitometry.

No significant change in the electrophoretic pattern was observed upon prolonged incubation periods (lanes c and d). However, more pro-SI, molecules were cleaved after 2 min of incubation with trypsin as assessed by comparing the labeling intensity of the products by densitometry (not shown). Essentially, similar results were obtained after 5 min of incubation. These data demonstrate that the cleavage of pro-SI, by trypsin is specific, unique to the generation of the two subunits, and instantaneous, since it occurs within 1 min.

**Fig. 3. Effect of pancreatic proteases on pro-SI, (A) and kinetics of the hydrolysis of pro-SI, by trypsin (B).** A, biopsy specimens were biosynthetically labeled with \(^{[35]}\)S-methionine for 4 h, homogenized, and divided into four equal samples: one sample was treated with 0.5 mg/ml trypsin (T) (lane b), a second with 0.5 mg/ml chymotrypsin (C) (lane c), and a third aliquot with 60 units/ml elastase (E) (lane d). The treated aliquots and the fourth untreated control (lane a) were incubated for 15 min at 37 °C. The reaction was stopped as indicated under “Experimental Procedures” and further processed for immunoprecipitation with anti-SI-antibodies. The immunoprecipitates were analyzed by SDS-gel electrophoresis and fluorography. B, four biopsy specimens were biosynthetically labeled for 4 h with \(^{[35]}\)S-methionine, homogenized in 4 ml, and divided into four samples each containing 1 ml equivalent to one biopsy sample. The aliquots were prewarmed to 37 °C, and trypsin was added at 0.5 mg/ml final concentration. Incubations were performed for 1, 2, and 5 min. SI was immunoprecipitated and subjected to SDS-gel electrophoresis and fluorography. The fluorograms were evaluated by densitometry.

**N- and O-Glycosylation of pro-SI and the Subunits S and I**

To determine the size, nature (high mannose/complex type), and mode of linkage (N-linked/O-linked) of the glycan units present on pro-SI, and the two subunits the following approaches were undertaken: 1) enzymic deglycosylation with endo H and endo F/GF of \(^{[35]}\)S-labeled pro-SI, S, and I and 2) determination of the binding capacity of sucrase-isomaltase to *H. pomatia* lectin and lentil lectin.

**Deglycosylation of pro-SI, S, and I with Endo H, Endo F/GF, and TFMS**—To determine the size of the endo F/GF product(s) of pro-SI, \(\text{BBM}\), and IM were isolated from \(^{[35]}\)S-homogenates of biopsy samples labeled for 4 h utilizing the Ca\(^{2+}\) precipitation procedure. SI was immunoprecipitated from these fractions and subsequently treated with endo F/GF. As shown in Fig. 4, SI purified from the brush border membrane fractions revealed predominantly pro-SI, (lane e) which was converted upon treatment with 1 unit of endo F/GF to an \(M_r\) = 205,000 species (lane f). No other digestion products were identified when higher endo F/GF concentrations were used. SI purified from the intracellular membrane fraction revealed both pro-SI, and pro-SI\(\text{h}\) (lane c). Endo F/GF digestion of these bands generated the \(M_r\) =
205,000 and 185,000 species (lane d). These results clearly show that the \( M_r = 205,000 \) species is the endo F/GF-form of the \( M_r = 245,000 \) protein. Assuming that pro-SI carries exclusively N-linked oligosaccharides, then deglycosylation of pro-SI, as well as pro-SIh, with endo F/GF should generate polypeptides of similar apparent molecular weights (\( M_r = 185,000 \)). Since this was not the case, we conclude that pro-SI contains in addition to N-linked carbohydrates endo F/GF-resistant glycan units which are most likely O-linked to serine or threonine residues.

To test this hypothesis, we treated SI purified from labeled biopsy samples (15 min or 4 h) with TFMS. This reagent, introduced by Edge et al. (41), is efficient in cleaving O-glycosyl bonds. It is therefore suitable for investigating whether further deglycosylation of endo F/GF-resistant forms of glycoproteins can be achieved. SI was treated with TFMS prior to or after endo F/GF digestion. In both cases, similar results were obtained. Fig. 5 shows that digestion of the pro-SI with endo H (lane b) and TFMS (lane c) gave digestion products of similar \( M_r \). The polypeptide of \( M_r = 185,000 \) was also revealed when SI isolated from 4-h labeled biopsy samples (containing pro-SIc and pro-SId) (lane d) was treated with TFMS (lane e). These results indicate that pro-SIc is not O-glycosylated and bears exclusively N-linked carbohydrate residues, since similar digestion products were obtained with endo H, endo F/GF (see also Fig. 1), and TFMS, whereas the mature species, pro-SIm, is N- as well as O-glycosylated. The difference in \( M_r \), between the endo F/GF- and TFMS-treated pro-SI (\( M_r = 205,000 \) versus 185,000) can therefore be attributed to the presence of approximately 20 kDa of O-linked sugars on serine or threonine residues of the mature molecule.

To assess the size and nature of glycan units of the sucrase and isomaltase subunits, homogenates of 4-h labeled biopsy specimens were treated with trypsin, aolubilized, and immunoprecipitated with anti-SI-beads. The immunoprecipitates were further hydrolyzed with endo H and endo F/GF. Fig. 6A, shows that at least one of the glycan units on the S, subunit was not converted to the complex type. In fact, treatment of sucrase-isomaltase immunoprecipitates with endo H resulted in a slight shift in \( M_r \) of the S, subunit (denoted by the open triangle in lane c) but not of L, The partial sensitivity of pro-SI to endo H is therefore accounted for by the presence of at least one high mannose chain on the S, molecule.

Digestion with 1 unit of endo F/GF converted the L, and S, subunits to polypeptides of \( M_r = 124,000, 109,000, 104,000, 99,000, \) and 94,000 (Fig. 6A, lane d). To determine which of these correspond to the endo F/GF forms of L, and S, we performed endo F/GF digestions on the individual subunits after excision from Coomassie Blue-stained gels. Thus, endo F/GF treatment of L, generated the \( M_r = 124,000 \) species, and S, was converted to polypeptides ranging in \( M_r \) between 109,000 and 94,000 (the results of the electrophoretic analysis were essentially similar to those shown in Fig. 9B (see below)). By contrast, one deglycosylated form of S, was revealed when SI was treated with TFMS. In fact, the \( M_r = 94,000 \) species was the only polypeptide among the components of endo F-treated S, detected, indicating that the other three endo F/ GF forms were converted to this form (Fig. 6B, lanes c and d). The L, subunit was shifted by TFMS to \( M_r = 111,000 \) (Fig. 6B, lanes c end d). Treatment of the individual subunits with TFMS confirmed that the \( M_r = 111,000 \) and 94,000 polypeptides are the totally deglycosylated forms of the L, and the S, respectively (results not shown). That the \( M_r = 111,000 \) and 94,000 forms are not artifactual products of TFMS treatment is internally governed by the deglycosylation of pro-SI and pro-SIh to the \( M_r = 185,000 \) polypeptide (lane b in Fig. 6B is shown to compare the size of the endo H-treated pro-SIh with that of the TFMS product). Prior hydrolysis of SI with endo

\[ \text{It should be noted that the slightly diffuse pattern of the } M_r = 94,000 \text{ form (and to a lesser extent the } M_r = 111,000 \text{) is not due to the presence in these bands of other molecular species of close } M_r. \text{ It possibly results from treatment of the slab gel with the fluorographic reagent and the low gel concentration (5%), since TFMS treatment of nonlabeled SI revealed on Coomassie Blue staining sharp bands on 6% slab gels (data not shown).} \]
tated molecules from permeabilized and trypsin-treated chains N-linked to sucrase and isomaltase, immunoprecipitated. In contrast, apparently one homogeneous population of the detergent extracts and treated with endo H were treated with trypsin. SI was immunoprecipitated from these aliquots and digested with concentrations of endo F/GF varying between 2–100 milliunits and with 1000 and 1500 milliunits. Samples were subjected to SDS-gel electrophoresis and fluorography. The figure is composed of three gels (0–20; 20–50 milliunits; and 60–100 milliunits, and 1000 and 1500 milliunits). The gels were run under identical conditions. The observed shifts are indicated by open triangles (I), solid triangles (S), and solid circles (pro-SI). The band patterns corresponding to endo F/GF concentrations of 50 and 60 milliunits were quite superimposable except for a faint band (denoted by the solid triangle) and major at 1000 and 1500 milliunits. The two bands observed below I and S, at 0 milliunits (the 118- and 113-kDa polypeptides) correspond to the core-glycosylated forms of isomaltase and sucrose which have resulted from cleavage of pro-SI, made accessible to trypsin upon permeabilization of the inside-out membrane vesicles.

FIG. 6. Effect of endo H and endo F/GF digestion on pro-SIh, pro-SI, and S, with TFMS (A). A, biopsy specimens were labeled with [35S]methionine for 4 h, and the homogenates were treated (lanes b–d) or not treated (a) with trypsin. SI was immunoprecipitated and treated with endo H (3 mIU) (lane c) or endo F/GF (1 unit) (lane d). Samples were analyzed by SDS-gel electrophoresis and fluorography. The slight reduction in the apparent molecular weight of S, upon endo H treatment is denoted by the open triangle in lane c. B, 35S-homogenates from biosynthetically labeled biopsy specimens (4-h labeling) were treated with trypsin. SI was immunoprecipitated from the detergent extracts and treated with endo H (lane b), TFMS (lane c), and TFMS after endo F/GF digestion (lane d). Treated and untreated (lane a) samples were analyzed by SDS-gel electrophoresis and fluorography.

F/GF was not required to achieve complete deglycosylation with TFMS, as shown by the similar digestion patterns of endo F/GF-treated or nontreated SI (Fig. 6B, lanes c and d).

In summary, these data show that the I, as well as the S, subunits contain O-linked oligosaccharides. The complexity of the endo F/GF pattern of S, is indicative of the existence of heterogeneous populations of S, molecules varying in their content of O-linked carbohydrates with most O-glycosylation on those molecules whose endo F/GF product is represented by the M, = 109,000 form. The M, = 94,000 species, on the other hand, is the endo F/GF digestion product of a set of molecules containing exclusively N-linked complex carbohydrates. In contrast, apparently one homogeneous population of I, molecules can be characterized, since only one endo F/GF-component (the M, = 124,000 form) was identified.

In an attempt to assess the number of carbohydrate side chains N-linked to sucrase and isomaltase, immunoprecipitated molecules from permeabilized and trypsin-treated 35S-labeled homogenates were digested with increasing amounts of endo F/GF (2–100 milliunits) to remove successively the various complex N-linked glycan units.

Fig. 7 shows that each subunit, S, as well as I, gave eight different intermediate forms at endo F/GF concentrations ranging between 2 and 70 milliunits. The digestion patterns remained essentially the same at higher endo F/GF concentrations (100–1500 milliunits). Consistent with the fact that endo F/GF cleaves N-linked carbohydrate side chains at the N-glycosyl linkage between N-acetylgalactosamine, galactose, and sialic acid (45).

The specific and strong interaction of H. pomatia lectin with N-acetylgalactosamine, galactose, and sialic acid (45) renders this lectin useful in the demonstration of O-linked oligosaccharides on glycoproteins.

We preferred to use in these experiments 35S-labeled homogenates rather than nonlabeled tissue, as dose-dependent deglycosylation of nonlabeled SI has imposed several technical problems related to the inadequate amount of the molecule remaining after digestion with endo F and precipitation with trichloroacetic acid.
TABLE I
Sucrase-isomaltase species and their deglycosylated forms

| Molecule                        | kDa | Deglycosylated forms | kDa |
|--------------------------------|-----|----------------------|-----|
| High mannosyl (pro-SIh)        | 210 | Endo H: 155          |     |
|                                |     | Endo F/GF: 185       | 185 |
| dNM-form*                      | 212 | NA                   | 185 |
| Complex glycosylated           | 245 | Endo H: 243          | 205 |
| (pro-SL)                       |     | Endo F/GF: 185       | 185 |
| Tunicamycin-form (pro-SI)      | 185 | Endo H: 185          |     |
|                                |     | Endo F/GF: 185       | 185 |
| Isomaltase-complex glycosylated| 145 | i45                  | 124 |
| (L)                            |     | TFMS: 109, 104, 94   |     |
| Sucrease-complex glycosylated  | 130 | Endo H: 128          | 99  |
| (S)                            |     | TFMS: 99, 94         |     |

* Pro-SI form revealed when labeling of biopsy specimens was performed in the presence of 1-deoxynojirimycin (dNM).

"These data are not presented under "Results."

Fig. 8. Binding of pro-SIh and pro-SIc to lectins. Biopsy specimens were biosynthetically labeled for 4 h with [35S]methionine, homogenized, and solubilized with 1% Triton X-100. The detergent extracts were divided into two equal samples and passed through lentil lectin-Sepharose or H. pomatia lectin-Sepharose. The columns were eluted with sugars specific for these lectins, the eluates dialyzed against the homogenization buffer and subsequently immunoprecipitated with anti-SI-antibodies. a, SI immunoprecipitated from eluates of the lentil lectin column; b, SI immunoprecipitated from eluates of the H. pomatia lectin column.

Crucial for the binding of glycoproteins containing these sugar types is removal of sialic acid to expose galactosyl/N-acetyl-
galactosaminy1 residues. However, sucrase-isomaltase iso-
lated from the adult small intestine has been shown not to contain sialic acids (26) and neuraminidase-treated and non-
treated SI bind equally to peanut agglutinin and SI-antibodies. Fig. 8 shows that pro-SIh was precipitated from the lentil lectin eluates (lane a) but not from those of H. pomatia lectin (lane b). Pro-SIc, was found in both eluates (lanes a and b). These data confirm that pro-SIh, is rich in mannosyl residues and show that pro-SIh does not contain co-
translationally added N-acetyl-D-galactosamine. In contrast, since it bound to H. pomatia lectin, pro-SIc is O-glycosylated and this type of glycosylation occurs later in the biosynthesis, most likely in an early region of the Golgi apparatus.

To obtain more information on the occurrence of O-glyco-
sylation sites on both subunits of SI, we investigated the binding of SI to H. pomatia lectin. For this purpose brush border membranes prepared from nonradioactive tissue (FII) were solubilized and run on H. pomatia lectin-Sepharose. The bound glycoproteins were eluted with N-acetyl-D-galactosa-
mime, immunoprecipitated with anti-SI-antibodies, and the precipitates were analyzed by SDS gel electrophoresis. Fig. 9A, lane b, shows that SI bound to H. pomatia lectin since it was detected in the eluates, thus providing further evidence for the occurrence of O-linked carbohydrates on the SI mole-
ecule. To show that the O-linked glycans are alone responsible for the binding of SI to H. pomatia lectin and to rule out any possible nonspecific binding or weak interaction which may originate from galactose residues of the N-linked glycan units, SI was purified from brush border membranes, digested with endo F/GF, and run on H. pomatia lectin-Sepharose. The molecules retained by the column revealed molecular species identical to those of endo F-treated SI used as control, indicating that endo F/GF-treated SI specifically reacted with H. pomatia lectin (Fig. 9A, lanes c and d).

The strong association between sucrase and isomaltase is well documented (7-9). This is also confirmed by the ability of monoclonal antibodies directed against different epitopes on either the sucrase or isomaltase molecules to immunopre-
cipitate similar molecular species (33). It is therefore reason-
able to assume that, for binding of SI to H. pomatia lectin to take place, the presence of O-linked sugars on one of the two molecules is sufficient. To corroborate, however, that both S, and I, contain sites reactive with H. pomatia lectin, the individual subunits of SI were passed through an H. pomatia lectin column after or without endo F/GF treatment. Electro
phoretic analysis of the bound material demonstrated by silve
staining of the gel that L, S,, as well as their correspondin
deoxy sugars bound to the lectin, indicating that bot subunits bear O-linked oligosaccharides (Fig. 9B). Finally TFMS treatment of I, and S, abolished completely the bindin
capacity of these molecules to H. pomatia lectin, demonst
rating that O-glycosidically linked glycans are responsible for the binding of SI to the lectin (data not shown).

Posttranslational Processing of SI

To identify more clearly the core-glycosylated intermediates of SI, biopsy specimens were labeled for 10 min with [35S]methionine and simultaneously treated with 5 mM dNM, a specific inhibitor of endoplasmic reticulum-bound glucose dase I (48). SI was immunopurified and a portion of the immunoprecipitate was treated with endo H. Under the conditions, the core-glycosylated precursor of SI was detect
d as a species at M, = 212,000 (Fig. 10, lane a); thus slight
higher than the polypeptide observed after short pulse labeli
in the absence of the inhibitor (Fig. 10, lane b). The electro
phoretic mobilities of both glycopeptides after endo H digi
tion remained unchanged (lanes c and d), indicating that t observed difference in the M, is accounted for by the sligh
clearer glycan units found on the polypeptide backbone
the presence of dNM. This shows that the M, = 212,000 polypeptide represents the initial co-translationally glyco
lated precursor of SI which undergoes trimming of the linked oligosaccharide units to yield the M, = 210,000 form.

To investigate the processing and subsequent maturat

H. Naim, unpublished experiments.

Fig. 9. Translation of S. and I. in brush border membranes. A: Brush border membranes (lanes a and b) were labeled with [35S]methionine for 10 min and treated with endo H or endo F/GF before electrophoresis. The gel was silver 

Fig. 10. A, Western blotting of SI and I to lectins. The lectin was incubated with SI, I, or I, prior to electrophoresis on a 12% gel. The lectin 

Fig. 11. A, Analysis of SI and I on a 12% gel. The gel was stained with silver to detect glycans. B, Western blotting of SI and I to lectins. The lectin was incubated with SI, I, or I, prior to electrophoresis on a 12% gel. The gel was silver stained to detect glycans.
of SI, pulse-chase experiments with \(^{35}\text{S}\)methionine combined with endo H treatments of immunoprecipitated SI were performed. The endo H-sensitive high mannose form of SI (pro-SIH) already appeared as a strong band after 10 min of pulse (Fig. 11, lane a). It could still be detected clearly after 240 min into the chase (Fig. 11, lane o). The fact that the \(M_\text{r} = 212,000\) form was not detected during the early stages of chase is suggestive of a rapid trimming of glucose residues from the core sugars on the precursor molecule. After 60 min of chase, a faint band corresponding to pro-SI was revealed (lane i). The intensity of this band became stronger upon prolonged chase periods indicating that more high mannose precursors were processed. However, complete conversion to

this mature form was not achieved after 4 h (lane o) or even after 18 h of chase (not shown).

The rate of transport from the rough endoplasmic reticulum to the Golgi was probed by the conversion of the processed molecule to endo H resistance. Thus, about 105–110 min were required for conversion of half of the high mannose precursor to the complex glycosylated, endo H-insensitive form (Fig. 11). To demonstrate that this processing takes place in the Golgi vesicles, pulse-chase experiments were undertaken in the absence of the carbohydrate ionophore monensin, whose site of action is the Golgi apparatus (49). Fig. 12 depicts the results of this experiment. As expected, in the presence of monensin, pro-SIH was not converted to pro-SI, after 120 min of chase (lane h), whereas in the absence of monensin it did (lane g). Similar results were obtained after 180 min of chase. Later into the chase, a broad band was detected of \(M_\text{r} = 210,000–230,000\) indicating that some processing of pro-SI has occurred but complete conversion to pro-SI, was inhibited by monensin. These data show that monensin greatly inter-
null
the transferred and further processed molecule to endo H resistance. This is also demonstrated by the unusual existence of a significant proportion of pro-SI, even after 4 and 18 h (not shown) of chase. The transport rate from the RER to the Golgi does not seem to be influenced by early modifications of the co-translationally added sugar chains. That DNM treatment of cultured biopsy samples produced precursor molecule of slightly higher apparent molecular weight \((M_r = 212,000)\) than pro-SI, whereas in the absence of the reagent and during the very early stages in the pulse-chase experiments similar molecules were not detected, is indicative of rapid processing of the core oligosaccharides. Similar data were obtained when the biosynthesis of lactase-phlorizin hydrolase, another disaccharidase of the intestinal brush border membrane, was investigated (37). Thus, the pulse-chase experiments with lactase-phlorizin hydrolase revealed slow conversion to the final endo H-resistant forms which occurred within 2 h of chase.

In experiments not shown, the transport kinetics of two brush border hydrolases dipeptidylpeptidase IV and aminopeptidase N were found to proceed 3- and 4-fold faster than those of SI (and also lactase-phlorizin hydrolase) from the RER to the Golgi. In spite of slight differences in the absolute transit times obtained, our data are consistent with those of Hauri et al. (33) in regard to the general concept of the occurrence of two classes of brush border membrane molecules which migrate at different rates within the small intestinal epithelial cells. A comparison with the conversion rates of a large number of membrane and secretory glycoproteins in a variety of cell systems favors classification of SI (and also lactase-phlorizin hydrolase) into a category of slow moving proteins. In accordance with the receptor-mediated transport mechanism (55, 56), SI may not bind avidly to the putative transport receptor and likely moves in a bulk-phase fashion in the lumen of transport vesicles. This might explain why a considerable proportion of the SI precursor remained in an endo H-sensitive form as a consequence of transport arrest and accumulation at the RER. It cannot be the large size of the molecule which caused the partial processing of the high mannose precursor; otherwise aminopeptidase N, also a large brush border membrane molecule, would have been processed at a rate similar to SI, and that was not the case (33).

The movement of the sucrose-isomaltase molecule within the Golgi vesicles and the accompanying covalent modifications appear to take place rapidly since intermediates of the co-translationally added sugar chains. That DNM treatment of cultured biopsy samples produced precursor molecule, contain N-linked as well as O-linked oligosaccharides. From the deglycosylation data with endo F/F, TFMS, we propose that the mature sucrose (S) subunit exists as a number of populations (at least four) which bear similar numbers of N-linked glycans (about eight) but differ in their content of O-linked oligosaccharides. In contrast, the mature isomaltase species (L) does not show a heterogeneous pattern of glycosylation.

The variability in the O-glycosylation of the sucrose subunit and the uniformity of that of isomaltase can be explained in two ways. Either by virtue of its location within the pro-SI molecule, all the O-glycosylation sites of isomaltase are clustered or distributed in such a way that they are more available than those of the sucrose molecule for the addition of O-linked glycans, or the heterogeneous cell population in the biopsy specimen gives rise to different levels of N-acetylglactosaminyltransferase which will add N-acetyl-D-galactosamine mainly to the most accessible O-glycosylation sites, and these are likely found on the isomaltase subunit. The first possibility offers, however, a more plausible explanation of our results, since it can be better interpreted in terms of the structural model of SI evolved from a number of studies which dealt with the association of the molecule with the membrane and the implication of this association on its biosynthesis. It has become apparent from these studies that sucrose-isomaltase is anchored to the microvillar membrane by a hydrophobic NH-terminal segment of the isomaltase subunit (12, 13). The synthesis of the precursor molecule starts with the isomaltase domain and ends with the sucrose subunit, implying that the carboxyl terminus of the pro-SI molecule is that of sucrose. More recently, Hunziker et al. (14) were able to predict the primary structure of rabbit pro-SI from the sequence of a nearly full length cDNA. Interestingly, a serine/threonine stretch in the isomaltase region may be located in close proximity to the membrane. From the sequence of pro-SI and the glycosylation data presented here, it can be hypothesized that the position of the O-glycosylation sites with respect to the carboxyl terminus is of paramount importance in the processing of O-linked glycan units and their subsequent presentation in the mature glycoprotein. Thus, some potential O-glycosylation sites may only circumstantially be made available during the concomitant and rapid processing of N- and O-linked carbohydrates. The O-glycosylation sites which do not always receive O-linked oligosaccharides are likely located in distal regions in the molecule or clustered at the COOH terminus. In accordance with the structural model proposed by Hunziker et al. (14), and extrapolating to human intestinal pro-SI, processing of O-linked sugars in the isomaltase region may occur first and involve all potential O-glycosylation sites, whereas the O-glycosylation sites of the sucrose subunit are located farther towards the COOH terminus and are not always accessible to the addition of O-linked sugars. This would explain why the mature sucrose subunit, in contrast to isomaltase, displays a heterogeneous pattern of O-glycosylation. The positioning of sucrose within the enzyme complex might also explain why at least one chain of the N-linked high mannose type remains unprocessed to the complex form. The validity of these interpretations will ultimately need detailed analyses of the carbohydrate structures and assessment of the location of the glycosylation sites on sucrose and isomaltase. We are currently trying to do this.

Consistent with the results shown in this paper, the sequence of events which the initial translation product undergoes during the biosynthesis can be dissected into: (i) rapid cotranslational glycosylation and trimming at the site of synthesis in the RER; (ii) slow transport to the Golgi where processing of N-linked and addition of O-linked glycans takes place; (iii) translocation after maturation to the brush border membrane and rapid extracellular cleavage by trypsin to two subunits. Since it is heavily O- and N-glycosylated, sucrose-isomaltase provides a good model for studying the temporal relationship between O-glycosylation and other well defined processing events involved in the biosynthesis of membrane proteins. The most interesting aspect in this regard is the differential O-glycosylation of two regions within a large precursor molecule.

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