Cell-free Synthesis, Membrane Integration, and Glycosylation of Pro-sucrase-isomaltase*

Paola Ghera, Peter Huber, Giorgio Semenza, and Hans Wacker

From the Laboratorium für Biochemie der Eidgenössischen Technischen Hochschule, CH 8092 Zürich, Switzerland

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Cell-free translation of total RNA from rabbit intestinal mucosa in a rabbit reticulocyte lysate, after immunoprecipitation with antibodies directed against sucrase-isomaltase, yielded a polypeptide of 200 kDa, which was identified as pro-sucrase-isomaltase. Addition of dog pancreatic microsomal vesicles to the translation system resulted in the appearance of an additional 220-kDa polypeptide. The 220-kDa polypeptide was associated with the membranes in a way that made it inaccessible to proteolysis; this protection was abolished by lytic detergent concentrations, indicating that the polypeptide was segregated into the microsomal vesicle. The 220-kDa polypeptide was glycosylated as evidenced by its being bound to concanavalin A-Sepharose and eluted with α-methyl-D-mannopyranoside. The increase in apparent molecular mass (~20 kDa) of the primary translation product upon translocation was due to the addition of carbohydrate; treatment of the 220-kDa polypeptide with endo-β-N-acetylglucosaminidase H increased its electrophoretic mobility to that of the 200-kDa polypeptide which was obtained in the absence of membranes. Partial N-terminal amino acid sequence of a translation product labeled with [3H]Leu in the absence of membranes revealed that Leu was incorporated into identical positions as in the final (pro)-sucrase-isomaltase, thus indicating the lack of a transient signal peptide.

The small intestinal sucrase-isomaltase complex is synthesized, glycosylated, and inserted into the membrane as a gigantic single polypeptide chain (pro-sucrase-isomaltase) which is subsequently processed by pancreatic proteases acting from the luminal surface to yield the final heterodimeric complex (1–5). We achieved the translation of pro-sucrase-isomaltase in vitro from total small intestinal RNA (6). Two bands precipitating with anti-sucrase-isomaltase antibodies and having apparent Mr values of 220,600 and 200,000, respectively, were found to be synthesized in the presence and/or in the absence of dog pancreas membranes. Much information on the biosynthesis of intestinal brush border hydrolases has been accumulated using organ culture (for a review see Ref. 7). However, the cell-free (and thus potentially better defined) in vitro translation system has only been used, in addition to sucrase-isomaltase, for the study of the biosynthesis of aminopeptidase N (8, 9) and γ-glutamyltransferase (10, 11).

The purpose of the present paper was to investigate (i) how related to one another the 200- and 220-kDa bands are; (ii) whether the products of the in vitro translation precipitating with anti-sucrase-isomaltase antibodies are segregated into the intravesicular space of dog pancreas membranes; (iii) whether the segregated product(s) are glycosylated; and (iv) whether pro-sucrase-isomaltase is originally made with a (transient) extension (i.e. as a “pre-pro-sucrase-isomaltase”).

**MATERIALS AND METHODS**

Guanidinium thiocyanate was from Fluka (Switzerland). All other chemicals were of analytical grade. L-[35S]Methionine (translation grade, 1000 Ci/mmol), L-[3H]leucine (147 Ci/mmol), and dog pancreas microsomes were from New England Nuclear or were prepared according to the method described in Ref. 12 and treated with micrococcal nuclease (12). Micrococcal nuclease and calf liver tRNA were from Boehringer (Federal Republic of Germany), amino acids were from Pierce Chemical Co. Proteinase K was purchased from Merck (Federal Republic of Germany); and endo-β-N-acetylglucosaminidase H (Endo-H) was from Miles. Tetraclain and cycloheximide were obtained from Sigma. Antiserum against rabbit sucrase-isomaltase had been raised in guinea pigs, and an IgG fraction free of proteolytic activity had been prepared by passing the serum over a column of CM Affi-Gel Blue (Bio-Rad) (13) followed by (NH4)2SO4 precipitation. Antiserum against SDS-denatured sucrase-isomaltase had been raised in a goat (6). Staphylococcus aureus, strain Cowan I, was heat inactivated (14) and fixed with glutaraldehyde according to the method described in Ref. 15.

Glassware was made nuclease free by heating overnight (180 °C); plastic ware was soaked in 0.2% aqueous diethylpyrocarbonate and autoclaved. All solutions were made up with water which had been treated with 0.2% diethylpyrocarbonate followed by autoclaving and autoclaved.

**Isolation of Total RNA**

Total RNA was extracted with 4 M guanidinium thiocyanate from fresh mucosal scrapings of rabbit small intestine and purified by repeated precipitation from 6 M guanidinium HCl with ethanol (16). The A260/A280 ratios were in the range of 2.

**In Vitro Protein Synthesis**

A cell-free translation system from rabbit reticulocyte lysate was used, and endogenous mRNA was digested with Ca2+-dependent micrococcal nuclease as described by Pelham and Jackson (17). Translations were performed for 120 min at 30 °C in the presence of 0.5–1 mM L-[35S]methionine or 2 mM of [3H]leucine, 0.77 ng of...
total RNA/ml of lysate, in the absence or in the presence (10 A260/ml lysate) of dog pancreas microsomes. Incorporation of radioactive amino acids into hot trichloroacetic acid-insoluble products was determined by spotting 2-μl aliquots on a strip of Whatman 3MM, 20 cm long (18), and processing them according to Mann and Novelli (19). The sections were cut apart and placed directly on a scintillation vial. For 3S the quenching on the filters was around 25%. For 3H the filter squares were wetted in a counting vial with 10 μl of H2O2, digested with 100 μl of NCS tissue solubilizer (Amersham Corp.) at room temperature under agitation, and then counted in 5 ml of a toluene-based scintillator.

**Immunoprecipitation of in Vitro Synthesized Pro-sucrase-isomaltase**

In *in vitro* synthesized pro-sucrase-isomaltase was immunoprecipitated using anti-sucrase-isomaltase antibodies and *Staphylococcus aureus* as described in Ref. 6 with minor modifications. Briefly, the translation mixture was made 1% in Triton X-100 and diluted 4 times with 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (Triton buffer), and 6 μg of sucrase-isomaltase was added as a carrier. A proteinase inhibitor mixture composed of 2.8 mg/ml aprotinin and 1 mg/ml each leupeptin, antipain, and chymostatin was added to make a final concentration of 20 and 8 μg/ml, respectively. Phenylmethylsulfonyl fluoride (100 mM in 2-propanol) and benzamidine were added (final concentration, 2 μM). After centrifugation at 100,000 g for 1 h, the pellet was resuspended in a very small volume of water. SDS (10%) and water (10%) was added to the sample and the sample was boiled for 2 min. After centrifugation in the Airfuge (100,000 g for 20 min) this supernatant was added to the previous one. The amount of SDS added to solubilize the pellet was chosen so that the final SDS concentration was 0.1%. This mixture was incubated overnight at 4°C with 3 μl of guinea pig anti-sucrase-isomaltase antibodies and then incubated for 1 h at room temperature under constant agitation on a tube rotator with 20 μl of a 10% (w/v) suspension of *S. aureus* in Triton buffer previously washed three times with the same buffer. After centrifugation through 0.3 ml of SDS-PAGE sample buffer containing 0.1% SDS and the acid (10 mM) used for labeling, another 20-μl portion of *S. aureus* was added and the incubation repeated as before. The combined *S. aureus* pellets were washed three times with 200 μl of Triton buffer containing 0.1% SDS.

Washed *S. aureus* pellets were resuspended in 10 μl of water; 50 μl of SDS-PAGE sample buffer (6% SDS, 1% mercaptoethanol, 14% glycerol in stacking gel buffer) was added and boiled for 5 min. *S. aureus* was removed by centrifugation, and the pellet was re-extracted twice as before. The combined supernatants were examined by SDS-PAGE. After staining of the gel with Coomassie blue the efficiency of immunoprecipitation could be assessed by comparing the staining of carrier sucrose-isomaltase recovered with the immunoprecipitate with that of a known amount of sucrose-isomaltase.

The bound material, whether synthesized in vitro or isolated with respect to microsomal vesicles) was determined by their ability to add pro-sucrase-isomaltase by incubating the sample gel pieces 15 min with 0.1 mM EDTA. 150 μl containing the solubilized material were diluted 30 times with 50 mM Tris-HCl, pH 7.2, 1% Triton, 150 mM NaCl, 5 mM MgCl2, 2 mM CaCl2, 0.1% SDS, and loaded on a column containing 0.1 ml of ConA-Sepharose 4B.

**Post-translational Assay for Segregation of Translation Products**

After *in vitro* translation, the location of the newly synthesized pro-sucrase-isomaltase chains (i.e. whether intracisternal or extracisternal with respect to microsomal vesicles) was determined by their ability to add pro-sucrase-isomaltase by incubating the sample gel pieces 15 min with 0.1 mM EDTA. 150 μl containing the solubilized material were diluted 30 times with 50 mM Tris-HCl, pH 7.2, 1% Triton, 150 mM NaCl, 5 mM MgCl2, 2 mM CaCl2, 0.1% SDS, and loaded on a column containing 0.1 ml of ConA-Sepharose 4B.

**Partial Characterization of Cell-free Synthesized Glycoproteins**

**Isolation of Glycoproteins by Affinity Chromatography on Concanavalin A-Sepharose 4B**—After translation and immunoprecipitation the *Staphylococcus* pellets were solubilized with three 50-μl portions of 50 mM Tris, pH 7.2, 3% SDS, 50 mM diethylenetriol, 6 M urea, 0.1 mM EDTA. 150 μl containing the solubilized material were diluted 30 times with 50 mM Tris-HCl, pH 7.2, 1% Triton, 150 mM NaCl, 5 mM MgCl2, 2 mM CaCl2, 0.1% SDS, and loaded on a column containing 0.1 ml of ConA-Sepharose 4B.

**Digestion with Endo-β-N-acetylglucosaminidase H**—For digestion with Endo-H the washed *Staphylococcus* pellets were solubilized by boiling for 2 min in 1% SDS, 1% 2-mercaptoethanol. The solubilized immunoprecipitates were made 0.2 M in sodium citrate buffer, pH 6.0, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 3 μg/ml aprotinin. Then Endo-H (final concentration, 0.1 unit/ml) was added, and the sample was incubated for 18 h at 37°C. One drop of tolune was added to prevent bacterial growth. A control was incubated under identical conditions, but lacking Endo-H. The reaction was terminated by addition of 4 volumes of cold (−20°C) acetone. After 6 h at −20°C the sample was centrifuged (45 min, 14,000 g) and the pellet dried under vacuum. The dried pellet was suspended in 10 μl of H2O and solubilized by boiling for 2 min in 50 μl of SDS-PAGE sample buffer.

3This second immunoprecipitation was carried out in order to reduce non-sucrose-isomaltase acid-sensitive radioactivity, which was still found in the first immunopellet. While not interfering in the SDS-PAGE analysis, this radioactivity would have interfered in radiosequencing.

4The first numeral denotes the total weight of monomer (acycliamide plus N,N-methylenebisacrylamide, g/100 ml, and the second numeral denotes the amount of N,N-methylenebisacrylamide expressed as a percentage (w/w) of the total amount of monomer (23).
RESULTS
Are the Translated Immunoprecipitable 200- and 220-kDa Bands Two Forms of Pro-sucrase-isomaltase?—Clearly, this question had to be answered unequivocally prior to any attempt to characterize them. Both bands are immunoprecipitated specifically with anti-sucrase-isomaltase antibodies, for preimmune serum does not produce any bands, and second, when immunoprecipitation is carried out with excess cold antigen both bands are diluted out (results not shown). To demonstrate by a different approach that both bands are indeed pro-sucrase-isomaltases we compared the V-8 protease peptide patterns obtained from (i) the 200-kDa immunoprecipitated band from translation mixtures without pancreas membranes (recovered from SDS-PAGE’s, such as that of Fig. 1, lane b), metastable [35S]Met labeled; (ii) and (iii) the 200- and 220-kDa bands from translation mixtures with pancreas membranes (also recovered from SDS-PAGE’s, such as that of Fig. 1, lane h, higher and lower band, respectively), also [35S]Met labeled; (iv) 125I-labeled (chloramine-T method) mature sucrase-isomaltase complex; and (v) 125I/TID-labeled mature sucrase-isomaltase complex. [125I]TID, a highly hydrophobic photolabel, labels the membrane-anchoring segment in sucrase-isomaltase complex, which is located in the N-terminal region of the isomaltase subunit (27, 28) and does not contain tyrosine residues. Thus, the peptide pattern from [125I]TID-labeled sucrase-isomaltase should be complementary to and different from that of chloramine-T-[125I]-labeled sucrase-isomaltase, which it is (compare Fig. 2, lanes a and b).

If the 200- and the 220-kDa [35S]Met-labeled bands from the translation mixtures are related to or identical with pro-sucrase-isomaltase (and if the V-8 peptides are sufficiently large), the [35S]Met peptide maps of the 220- and the 200-kDa bands should be similar to the sum of the peptide maps from [125I]TID-labeled plus chloramine-T-[125I]-labeled sucrase-isomaltase, which is indeed the case (Fig. 2). This observation, in addition to these bands precipitating specifically with anti-sucrase-isomaltase antisera and to them having apparent Mr in the “right” range, conclusively demonstrates that they are two forms of pro-sucrase-isomaltase.

Is the 220-kDa Band, Which Is Synthesized in the Presence of Microsomal Membrane Vesicles, Segregated into Them?—This higher Mr band (220 kDa) sediments together with the membranes whereas the smaller (200 kDa) does not; compare in Fig. 1, lane e (from the pellet of a 14,000 × g × 15-min centrifugation of a translation mixture in the presence of membranes) with lane d (from the supernatant).

When, after translation in the presence of membranes, the mixture is incubated with proteinase K, the 220-kDa band is not degraded (Fig. 1, lane g) unless lytic concentrations of detergent are also present (lane f). Thus, the polypeptide associated with the membrane is segregated into the intravesicular space. In contrast, the 200-kDa band, which, as mentioned above, does not sediment with the membranes, is completely degraded by proteinase K, also in the absence of any detergent (lane g). When translation is carried out in the absence of microsomal membranes and the mixture is then treated with proteinase K, the 200-kDa band is totally degraded (lane c, compare with the control in lane b).

Therefore, it seems clear that the 220-kDa band alone is associated with the dog pancreas membranes in a compartment or in a manner making it inaccessible to proteolytic degradation. In all likelihood the 220-kDa band is located in the intravesicular space and/or within the membrane itself, and this is in agreement with what is known about the biosynthesis of membrane proteins (29) and with the known positioning of sucrase-isomaltase and pro-sucrase-isomaltase (30).

From these results one can conclude that only that newly synthesized polypeptide with a migration corresponding to an apparent Mr, of 220,000 is translated into the intravesicular space of the microsomal vesicle. The apparent Mr of the 220-kDa band does not detectably change upon treatment with proteinase K of the microsomal vesicles, suggesting the absence of proteolytic degradation. In all likelihood the 220-kDa band is located in the intravesicular space and/or within the membrane itself, and this is in agreement with what is known about the biosynthesis of membrane proteins (29) and with the known positioning of sucrase-isomaltase and pro-sucrase-isomaltase (30). From these results one can conclude that only that newly synthesized polypeptide with a migration corresponding to an apparent Mr, of 220,000 is translated into the intravesicular space of the microsomal vesicle. The apparent Mr of the 220-kDa band does not detectably change upon treatment with proteinase K of the microsomal vesicles, suggesting the absence of proteolytic degradation. In all likelihood the 220-kDa band is located in the intravesicular space and/or within the membrane itself, and this is in agreement with what is known about the biosynthesis of membrane proteins (29) and with the known positioning of sucrase-isomaltase and pro-sucrase-isomaltase (30). From these results one can conclude that only that newly synthesized polypeptide with a migration corresponding to an apparent Mr, of 220,000 is translated into the intravesicular space of the microsomal vesicle. The apparent Mr of the 220-kDa band does not detectably change upon treatment with proteinase K of the microsomal vesicles, suggesting the absence of proteolytic degradation. In all likelihood the 220-kDa band is located in the intravesicular space and/or within the membrane itself, and this is in agreement with what is known about the biosynthesis of membrane proteins (29) and with the known positioning of sucrase-isomaltase and pro-sucrase-isomaltase (30). From these results one can conclude that only that newly synthesized polypeptide with a migration corresponding to an apparent Mr, of 220,000 is translated into the intravesicular space of the microsomal vesicle. The apparent Mr of the 220-kDa band does not detectably change upon treatment with proteinase K of the microsomal vesicles, suggesting the absence of proteolytic degradation.
and treated with V-8 proteinase (22). For comparison, samples of
220 kDa were cut from the gel, transferred to a second (14%) gel,
containing 0.1% SDS (see Fig. 1). The bands with apparent
molecular weight standards (ovalbumin (46), carbonic anhydrase
(140), sucrase subunit (120), phosphorylase (97), bovine serum
albumin (69)). The peptide with the lowest mobility (corresponding
to an apparent molecular weight of ~21,000) in lane b is also visible after a longer exposure on lanes a and c, but does not show up on the photograph.

Is the 220-kDa Membrane-segregated Pro-sucrase-isomaltase Glycosylated?—N-Glycosylation of proteins takes place co-translationally in rough endoplasmic reticulum membranes; “trimming,” re-glycosylation, and 0-glycosylations take place in the Golgi membranes (reviewed in Ref. 31). Since sucrase-isomaltase is a glycoprotein, containing both N- and O-glycosidically linked sugars (32, 33) and since it is known that dog pancreatic vesicles are capable of cotranslational N-glycosylation (12), we investigated whether the decrease in mobility of the segregated polypeptide chain (corresponding to an increase in the apparent molecular weight of around 20,000) was due to glycosylation. To this end we tested its binding ability to concanavalin A-Sepharose and its sensitivity to Endo-H.

As can be seen in Fig. 3 the band with the lower mobility which is segregated into the intravesicular space (Fig. 1) binds to ConA-Sepharose and can be eluted with 500 mM α-methyl-D-mannopyranoside (lane d). The bands with the higher mobilities obtained in the presence and absence of membranes are not retained by the column and can be recovered from the flowthrough volume (lanes b and c).

Further evidence that the larger apparent Mₙ of the band segregated into the microsomal vesicular space and retained by concanavalin A is indeed due to (core) glycosylation was obtained by digestion with Endo-H. Incubation with Endo-H increases the mobility of the upper band without affecting that of the lower one or that of the band synthesized in the absence of microsomal membranes; furthermore, after Endo-
H treatment all the bands have indistinguishable behavior in SDS-PAGE (apparent Mₙ = 200,000) (results not shown).

Is Pro-sucrase-isomaltase Synthesized as “Pre-pro-sucrase-isomaltase”?—That is, is pro-sucrase-isomaltase originally made with an N-terminal extension (signal peptide), and, if so, is this piece lost upon translocation? An answer to this question cannot be obtained simply by comparing the sizes of translation products (say, in SDS-PAGE) obtained in the presence or in the absence of microsomal membranes because of the very large Mₙ of pro-sucrase-isomaltase (an additional 20 amino acid residues would increase the Mₙ by approximately 1%) and because the polypeptide is glycosylated while being translocated. Therefore, we carried out a partial N-terminal sequence analysis of the 200 kDa in vitro translation product without microsomal membranes, using [³H]Leu as the radioactive amino acid. As can be seen from Fig. 4, [³H] Leu is incorporated into the in vitro translation product in positions corresponding exactly to the leucine residues of the N-terminal sequence of mature pro-sucrase-isomaltase (and of the isomaltase subunit) (5, 28). The small radioactivity peak at position 17 remains unexplained. It is possibly related to the background, which is high in this region. The close
match observed between the positions of the [3H]Leu incorporated in vitro (in the absence of microsomal membranes) and the position of leucine in the N-terminal region of mature pro-sucrase-isomaltase argue against the transient existence of a signal peptide.

**DISCUSSION**

The purpose of the present work was to investigate whether (i) the two polypeptides translated in vitro in a cell-free system in the absence or presence of microsomal membranes which immunoprecipitate with antibodies directed against "final" sucrase-isomaltase are two forms (about 200 and 220 kDa) of single-chain pro-sucrase-isomaltase and whether (ii) they (or one of them) segregate into the membranes according to the mechanism established for other much shorter membrane polypeptides. The unusual size of pro-sucrase-isomaltase was one of the motives of our interest in this very peptide.

The results clearly identify both high molecular weight bands as forms of pro-sucrase-isomaltase; in addition to having a molecular size in the proper (and fairly unusual) range, they precipitate with polyclonal antibodies directed against "final" sucrase-isomaltase (Fig. 1); also the peptide maps obtained from V8 protease treatment of the two bands (labeled with [35S]Met) correspond fairly well to the sum of the bands which are obtained from sucrase-isomaltase labeled in the (tyrosine-free) hydrophobic region with [125I]TID plus sucrase-isomaltase labeled at tyrosine groups with ['251]I (chloramine-T method). (In addition, the identical peptide maps pattern yielded by the 200-kDa (i.e. non-membrane associated) and the 220-kDa (i.e. membrane associated) pro-sucrase-isomaltases (Fig. 2) and the results of the N-terminal sequence analysis of the non-membrane associated (and thus presumably primary non-cleaved) in vitro translation product labeled with [3H]Leu (Fig. 4) suggest that the N-terminal sequence of the primary translation product and that of the mature in vivo product are identical. This would preclude the existence of a cleavable transient signal peptide; possibly, as suggested some years ago, the hydrophobic anchors of mature membrane hydrolases (11, 41-44) may act as signal also for pro-sucrase-isomaltase (28, 40, 41) and other brush border membrane hydrolases (11, 41-44) may act as signal also ("permanent insertion signal" (29)). In all fairness, in the last few years we favored the hypothesis of a cleavable signal, mainly due to the position of the N terminus of isomaltase (and pro-sucrase-isomaltase), which was thought to be extracellular. We now know, however, that this N terminus is located at the cytosolic side of the membrane.

Sucrase-isomaltase and pro-sucrase-isomaltase are anchored to the membrane via a highly hydrophobic segment located in the N-terminal region of the isomaltase subunit (or portion, respectively) (6, 30, 39). The identical peptide map pattern yielded by the 200-kDa (i.e. non-membrane associated) and the 220-kDa (i.e. membrane associated) pro-sucrase-isomaltases (Fig. 2) and the results of the N-terminal sequence analysis of the non-membrane associated (and thus presumably primary non-cleaved) in vitro translation product labeled with [3H]Leu (Fig. 4) suggest that the N-terminal sequence of the primary translation product and that of the mature in vivo product are identical. This would preclude the existence of a cleavable transient signal peptide; possibly, as suggested some years ago, the hydrophobic anchors of mature (pro)sucrase-isomaltase (28, 40, 41) and other brush border membrane hydrolases (11, 41-44) may act as signal also ("permanent insertion signal") (29). In all fairness, in the last few years we favored the hypothesis of a cleavable signal, mainly due to the position of the N terminus of isomaltase (and pro-sucrase-isomaltase), which was thought to be extracellular. We now know, however, that this N terminus is located at the cytosolic side of the membrane.

This dual role of an N-terminal hydrophobic anchoring peptide has been demonstrated for neuraminidase of human influenza virus (45). In the case of other brush border membrane proteins, there are various degrees of evidence that at least some, in addition to pro-sucrase-isomaltase, also carry a permanent insertion signal at the N-terminal region of the

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chain. The evidence is the strongest perhaps in the case of pro-γ-glutamyl transferase (10, 11) for which the fairly small length of the chain has allowed a reliable comparison of the size of the in vitro cell-free translation product (in the absence of membranes) with that of the translation product in the presence of membranes after Endo-H treatment. But also obtained by cDNA cloning and sequencing?

the main lines of a unifying Picture are emerging (for insertion signal located not far from their N termini. Thus, at the time of writing, to be endowed with a permanent aminopeptidases of membranes) with that of the translation product in the size of the biosynthesis of pro-sucrase-isomaltase has been recently carried out the Edman degradation of Fig. 7974.

Acknowledgments—We thank Prof. E. E. Rickli, Berne, for having carried out the Edman degradation of Fig. 4.

Note Added in Proof—Final evidence for the lack of a cleavable signal in biosynthesis of pro-sucrase-isomaltase has been recently obtained by cDNA cloning and sequencing.6

REFERENCES