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Biogenesis of intestinal plasma membrane: Posttranslational route and cleavage of sucrase–isomaltase*

(fucose labeling/putative enzyme precursor/membrane flow/late posttranslational processing/elastase)

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ABSTRACT **The biosynthesis *in vivo* of rat intestinal sucrase–isomaltase [a complex of sucrose α-glucohydrolase, EC 3.2.1.48, and oligo-1,6-glucosidase (dextrin 6-α-D-glucanohydrolase), EC 3.2.1.10] has been studied by following the incorporation of L-[6-^{3}H]fucose into the enzyme with time. Immunoprecipitation of sucrase–isomaltase from Triton-X-100-solubilized Golgi or basolateral membranes and subsequent polyacrylamide gel electrophoresis revealed the presence of an immunoreactive glycoprotein with an apparent molecular weight approximately twice that of the separated sucrase–isomaltase subunits, but no active subunits were found in these membranes. This glycoprotein was also found in the microvillus membrane in addition to the subunits of sucrase–isomaltase. Kinetic studies showed a maximal labeling of this glycoprotein in Golgi membranes at 15 min, in basolateral membranes at 30 min, and in microvillus membranes at 45 min and a half-life of less than 30 min in each membrane. However, the radioactivity of the sucrase–isomaltase subunits in the microvillus membrane reached a plateau after 60 min. These data suggest that sucrase–isomaltase is synthesized as a one-chain polypeptide precursor that is split into the subunits after its transfer to the microvillus membrane. Elastase (EC 3.4.21.11), but not trypsin (EC 3.4.21.4) or α-chymotrypsin (EC 3.4.21.1), split the putative precursor into two polypeptides that had electrophoretic behaviors similar to those of the active enzyme subunits. These studies suggest that pancreatic proteases may play an important role in the late posttranslational processing of sucrase–isomaltase *in vivo*.**

The membrane flow theory for the biogenesis of the plasma membrane assumes that there is a physical transfer of membrane components from the endoplasmic reticulum to the Golgi complex and subsequently to the cell surface (2). This concept, however, is still controversial, mainly due to the considerable differences in liquid and protein composition among the different membrane compartments (3). Much insight into membrane biogenesis has been provided recently by studies concerning the early posttranslational events in the biogenesis of viral glycoproteins (4–6), but the knowledge of the posttranslational fate of eukaryotic membrane proteins is still limited. In order to elucidate some of these mechanisms we have studied the biosynthesis of the sucrase–isomaltase complex [SI; sucrose α-glucohydrolase, EC 3.2.1.48, and oligo-1,6-glucosidase (dextrin 6-α-D-glucanohydrolase), EC 3.2.1.10] of the rat small intestinal microvillus membrane (MVM).

SI is an intrinsic membrane glycoprotein consisting of two similar subunits with apparent molecular weights of approximately 140,000 to 160,000 each; one subunit splits sucrose and maltose and the other hydrolyzes isomaltose and maltose (7). Studies of rabbit SI have shown that this enzyme is anchored to the MVM via a hydrophobic amino acid sequence near the NH_2 terminus of the isomaltase subunit with the sucrase subunit occupying a more peripheral position (8, 9). The anchoring of SI by means of an NH_2-terminal rather than a COOH-terminal region of only one of the two subunits is of particular interest because it is not easily explained by mechanisms currently accepted for the biogenesis of membrane glycoproteins (4, 6). It has been suggested on purely theoretical grounds that SI might be synthesized as a single-chain precursor and that its hydrophobic anchor might represent a noncleavable signal sequence (8–10).

The present report provides experimental evidence for part of this hypothesis. We have shown that rat SI is synthesized as a high molecular weight precursor that is cleaved into subunits in the MVM. *In vitro* studies suggest that pancreatic enzymes may play a role *in vivo* in this late posttranslational cleavage process.

MATERIALS AND METHODS

[^{3}H]Fucose Labeling and Subcellular Fractionation. Overnight fasted rats (150–200 g, Charles River Breeding Laboratories) were intraperitoneally injected with 500 μCi (1 Ci = 3.7×10^{10} becquerels) of L-[6-^{3}H]fucose (New England Nuclear) in 300 μl of saline each. Golgi membranes (GM, gradient fraction 20/30 + 30) and basolateral membranes (BLM, gradient fraction 40) were isolated from differentiated enterocytes [cell fraction 1–5 (11)] as described (12). MVM were isolated from mucosal scrapings (13). In the case of suckling rats the MVM were prepared according to Kessler *et al.* (14). For the kinetic studies GM and BLM were isolated from mucosal scrapings (15) in order to be able to compare the results directly with the result obtained from MVM. All membranes were resuspended in a small volume of distilled water and stored frozen (−20°C) for not longer than 3 weeks before use.

Immunoprecipitation. L-[6-^{3}H]Fucose-labeled membranes were mixed with Triton X-100 to a final volume of 550 μl and a final concentration of 1% (wt/vol). The solubilization mixture was sonicated three times for 1 sec and left on ice for 2 hr. This procedure solubilized 60–70% (GM or BLM) and 80–90% (MVM) of total membrane-associated [^{3}H]fucose, as measured by liquid scintillation counting. After centrifugation at 100,000 × *g* for 1 hr, the supernatants were immunoprecipitated with 50 μl of rabbit antibody (IgG fraction, 4 mg of protein per ml of phosphate-buffered saline) to purified rat SI (16) at 4°C overnight. To the GM or BLM, nonlabeled Triton-X-100-solubilized MVM containing 0.125 international unit of sucrase activity were added as a carrier prior to immunoprecipitation. The precipitates were centrifuged for 2 min at 8000 × *g* in an Eppendorf centrifuge and the supernatants were checked for complete immunoprecipitation by measuring sucrase activity. The precipitates were washed twice with 1 ml of 0.45% saline

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Abbreviations: SI, sucrase–isomaltase complex; MVM, microvillus membranes; GM, Golgi membranes; BLM, basolateral membranes; P, putative precursor of sucrase–isomaltase.

* Part of this work has been published in abstract form (1).

containing 1% (wt/vol) Triton X-100 and once with H_2O and were resuspended in 100 μl of H_2O by brief sonication.

Polyacrylamide Gel Electrophoresis and Measurement of Radioactivity. To the washed and resuspended immunoprecipitates was added 50 μl of three times concentrated sample buffer containing 50 mM dithiothreitol, and the samples were immediately heated to 100°C for 3 min. Electrophoresis was performed on 7½% polyacrylamide slab gels in the presence of sodium dodecyl sulfate (17). Radioactivity was visualized by fluorography of the dried gel (18) using Kodak RP Royal X-Omat films and an exposure of 7–21 days at −70°C. For the study of kinetics the radioactivity associated with the putative precursor (P) or the SI subunits was quantitated in the following way. One milligram of [^{3}H]fucose-labeled GM or BLM protein or MVM containing an equivalent of 0.125 international unit of sucrase activity was solubilized with Triton X-100 and immunoprecipitated with anti-SI, and the precipitates were separated by gel electrophoresis as above. The individual lanes from the Coomassie blue-stained gels were scanned at 560 nm wavelength, frozen with solid CO_2, and cut into 1-mm slices with a Mickle slicer. The slices were dissolved in H_2O_2 and radioactivity was measured by liquid scintillation counting (19). The radioactivities from slices containing either P or the SI subunits were combined. In the case of MVM, radioactivity was corrected for the actual amount of SI subunit protein on the gel as calculated from the area under the corresponding peaks of the protein scan.

Treatment of P with Pancreatic Proteases. [^{3}H]Fucose-labeled membranes containing 150,000 cpm (GM or BLM) or 100,000 cpm (MVM) were resuspended in 15 mM potassium phosphate buffer, pH 7.1, by brief sonication (three times, 1 sec) and incubated for 30 min at 37°C with bovine trypsin (EC 3.4.21.4) at a final concentration of 0.14 mg/ml, bovine α-chymotrypsin (EC 3.4.21.1) at 0.14 mg/ml, or porcine elastase (EC 3.4.21.11) at 18 units/ml (1 unit solubilizes 1 mg of elastin in 20 min at pH 8.8 at 37°C) in a New Brunswick gyrotory shaker (85 cycles/min). The enzyme reaction was stopped by adding soybean trypsin inhibitor at 0.24 mg/ml (final concentration) in the case of trypsin or phenylmethylsulfonyl fluoride at 1.87 mM in the case of chymotrypsin or elastase. Thereafter the incubation mixture was immediately cooled to 4°C, solubilized with Triton X-100 (1% final concentration), immunoprecipitated, and separated on slab gels. Alternatively, Golgi membranes were first solubilized with Triton X-100 for 2 hr on ice. After centrifugation for 1 hr at 100,000 × *g* the supernatants were treated with proteases and further processed as above.

Measurement of Microvillus Enzyme Activities and Protein. Sucrase, isomaltase, maltase (α-glucosidase, EC 3.2.1.20), lactase (β-D-galactohydrolase, EC 3.2.1.23), trehalase (α,α-trehalase, EC 3.2.1.28), alkaline phosphatase (EC 3.1.3.1), and aminopeptidase (EC 3.4.11.2) activities were measured by standard methods for these enzymes (20–23). Protein was determined with bovine serum albumin as a standard (24).

RESULTS

Demonstration of a Putative Precursor (P) of SI. Fluorograms of polyacrylamide gels run with immunoprecipitates from GM or BLM obtained 30 min after fucose labeling exhibited an immunoreactive glycoprotein (P) with an apparent molecular weight approximately twice that of the separated SI subunits (Fig. 1). It is noteworthy that no subunits were present in these two membrane compartments. P was virtually absent from these membranes after 3 hr. With immunoprecipitates from MVM the fluorograms were different: in addition to labeling of P, two radioactive bands comigrating with the SI subunits were found after 30 min (Fig. 1). While the intensities of these two bands became more pronounced, P was absent from the fluorogram at 3 hr. These findings suggested that P was a high molecular weight SI precursor that was subsequently split into the subunits in the MVM.

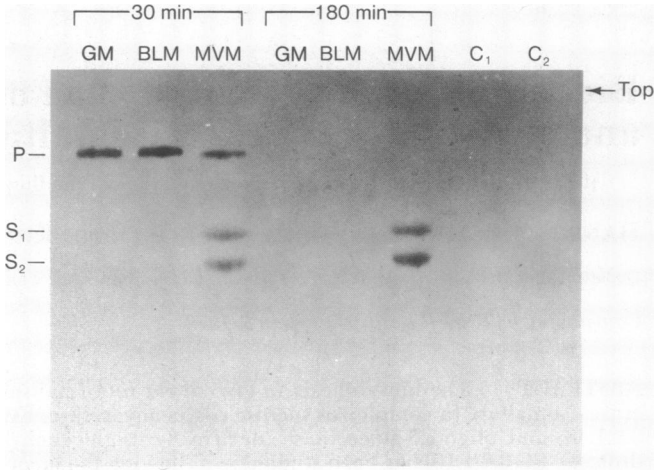

FIG. 1. Fluorogram of *in vivo* synthesized SI and its putative precursor. L-[6-^{3}H]Fucose-labeled GM or BLM containing 150,000 cpm and MVM containing 100,000 cpm were solubilized with Triton X-100 and immunoprecipitated with the SI antibody. The immunoprecipitates were separated on polyacrylamide gels in the presence of sodium dodecyl sulfate and reducing agents. The fluorogram shows the radioactivity patterns obtained after 30 and 180 min from GM, BLM, and MVM. C_1, control with MVM from 12-day-old suckling rats 60 min after fucose labeling (carrier immunoprecipitation); C_2, control with GM (30 min after fucose labeling), using a nonspecific antibody (IgG fraction of rabbit anti-human IgA). P, putative precursor of SI; S_1 and S_2, subunits of SI.

Specificity of SI Antibody. Because these studies were based on the specificity of the SI antibody, it was essential to carefully assess this antibody specificity. When the antibody was analyzed in Ouchterlony double diffusion tests with pure SI a single precipitation line resulted (16); quantitative immunoprecipitation with an unrelated antibody gave no visible precipitate with GM or any radioactive bands on fluorograms (Fig. 1). MVM from 12-day-old rats known to lack any intestinal sucrase or isomaltase activity (25) did not show any radioactive bands on fluorograms when immunoprecipitated with anti-SI (Fig. 1). With MVM from adult rats, the SI antibody precipitated 98% of sucrase, 92% of isomaltase, and 58% of maltase activities but not more than 1% of lactase, 2% of trehalase, 1% of alkaline

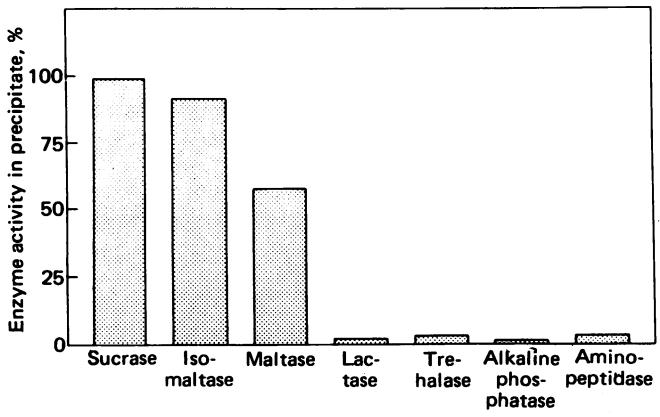

FIG. 2. Specificity of the SI antibody. Representative levels of activities of MVM enzymes in the washed immunoprecipitate.

phosphatase, and 3% of aminopeptidase activities (Fig. 2). Because two different enzyme complexes (i.e., SI and maltase–glucoamylase) are known to be able to split maltose in the rat small intestine (26), these findings corroborate the antibody specificity.

Flow Kinetics of P from Golgi to Surface Membranes. In order to validate the precursor–product concept, kinetics of flow of P from the Golgi complex to the surface membrane and its fate in the MVM were established quantitatively. Fig. 3 shows that P was maximally labeled in the GM after 15 min, in the BLM after 30 min, and in the MVM after 45 min. Its apparent half-life in these membranes was less than 30 min. In contrast, the radioactivity associated with the subunits of SI in the MVM was maximal after 60 min and remained virtually unchanged for the following 2 hr. These data indicate a transfer of P from GM to MVM. The rapid disappearance of P from the latter membrane, concomitant with the appearance of radioactivity in the SI subunits, supports a precursor–product concept. An unexpected but interesting finding was the association of P with the BLM. While this result could merely reflect contamination of this membrane fraction with Golgi elements, the fact that P in the GM was maximally labeled 15 min before that in the BLM suggests that cross-contamination cannot fully account for this observation. Indeed, a recent study (27) on the effect of colchicine on the redistribution of *in vivo* fucose-labeled intestinal membrane glycoproteins lends further support to the existence of an additional pathway. The findings of Quaroni *et al.* (27) suggest that approximately 60% of newly synthesized membrane glycoproteins are inserted from the Golgi complex into the BLM and may reach the MVM by a redistribution process.

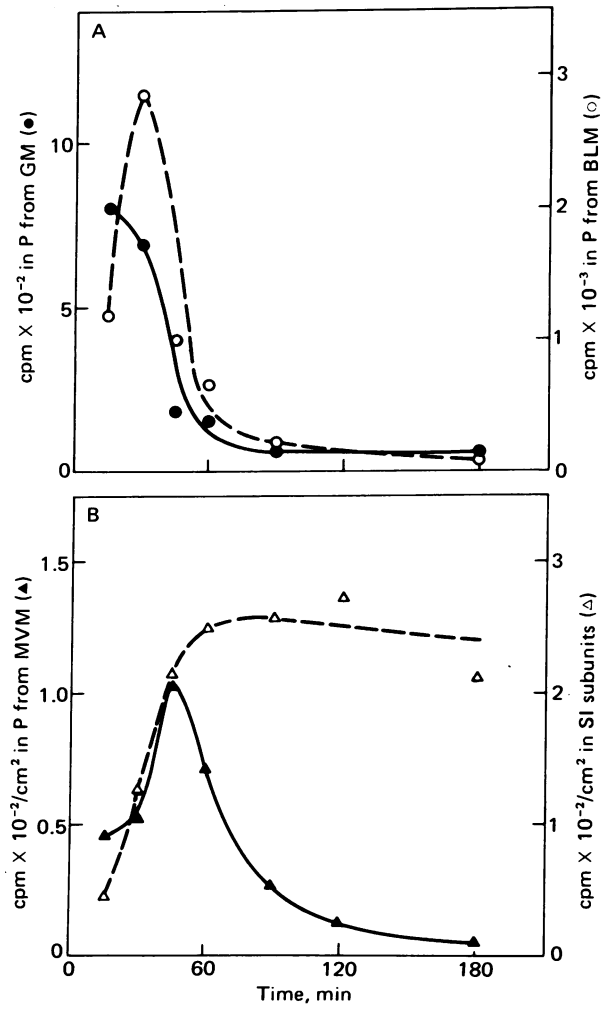

FIG. 3. Kinetics of the flow of putative SI precursor P from Golgi to surface membranes and appearance of newly synthesized enzyme subunits (S_1 and S_2) in the MVM. GM or BLM (*A*) and MVM (*B*) were isolated at various time intervals after the injection of L-[6-^{3}H]fucose. The solubilized membranes were immunoprecipitated with the SI antibody and the precipitates were separated on polyacrylamide slab gels. Total radioactivity in P or in the enzyme subunits was quantitated from gel slices.

Cleavage of P by Elastase *In Vitro*. To explore the cleavage mechanism of P into enzyme subunits, fucose-labeled GM were treated with subphysiological amounts of pancreatic proteases (for physiological concentrations see ref. 28). These enzymes have been reported to play an important role in the turnover of intestinal MVM proteins (28). With intact GM, trypsin and chymotrypsin produced no apparent splitting of P (Fig. 4, experiment B). However, treatment with elastase resulted in the appearance of two additional radioactivity bands, one exactly in the position of subunit 1 and the other running slightly behind subunit 2, concomitant with the virtual disappearance of P. To avoid the possible inaccessibility of the proteases to the inside of the membrane vesicles, the presumed site of the bulk of protein P, GM were solubilized with Triton X-100 prior to protease treatment (Fig. 4, experiment A). This approach led to similar results except that, once solubilized, P appeared to be somewhat less resistant to trypsin. As seen in Fig. 4, elastase also produced similar splitting with BLM. With MVM, elastase but not trypsin resulted in the disappearance of P (Fig. 4). Both elastase and trypsin increased the mobility of the slower migrating SI subunit slightly. However, elastase treatment did not generate any additional bands, suggesting an identical electrophoretic behavior of the cleavage products of P and the SI subunits.

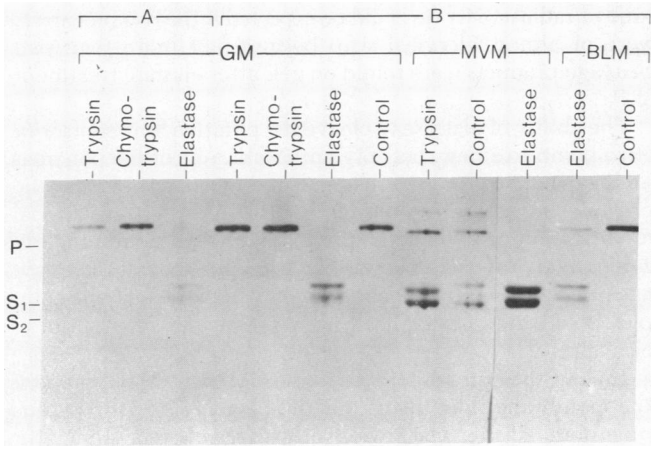

FIG. 4. Effect of pancreatic proteases on the putative SI precursor P from GM, BLM, and MVM. (Experiment A) L-[6-^{3}H]fucose-labeled GM (150,000 cpm) were solubilized with Triton X-100 and incubated for 30 min at 37°C with trypsin, chymotrypsin, or elastase prior to immunoprecipitation and gel electrophoresis. (Experiment B) Alternatively, intact GM (150,000 cpm), BLM (150,000 cpm), or MVM (100,000 cpm) were treated with proteases as above. The fluorogram shows the radioactivity pattern of the immunoprecipitates obtained 30 min (GM, BLM) or 45 min (MVM) after fucose labeling. The controls were incubated in the absence of proteases. The fluorogram is a composite of two separate slab gels aligned by an identical MVM control present in each gel.

DISCUSSION

The results of the present study suggest that P is the precursor of SI. We are aware of the pitfalls of interpreting data from experiments relying predominantly on antibody specificity. However, the following considerations strongly support a precursor–product concept for the biosynthesis of SI: (*i*) Our SI antibody has been prepared against highly purified SI and meets the generally accepted criteria for monospecificity. (*ii*) In both GM and BLM, P was the only glycoprotein reacting with the antibody. In particular, these membranes apparently did not contain any SI subunits as revealed by quantitative immunoprecipitation or any other glycoprotein comigrating on gels with the enzyme subunits when the whole pattern of membrane glycoproteins was analyzed (27). (*iii*) The kinetics of transfer of P to the cell surface and the rapid decrease of its radioactivity in the MVM, concomitant with the increase of radioactivity in the SI subunits, further support a precursor–product concept. (*iv*) Considerable resistance to trypsin and chymotrypsin, a well-known property of SI, was also found for P. (*v*) *In vitro* cleavage of P from MVM by elastase showed an identical electrophoretic behavior of its cleavage products with the enzyme subunits. With GM or BLM the *in vitro* cleavage of P by elastase generated two peptides, one of which exactly comigrated with subunit 1 whereas the other one ran slightly behind subunit 2. This might reflect the presence of an additional peptide sequence in the latter fragment that is split off during normal processing of the precursor in the MVM.

The demonstration of a putative high molecular weight precursor provides an experimental basis for the hypothesis of Semenza (8, 10) on the mechanisms of SI biosynthesis. Semenza speculated that the related biological control of sucrase and isomaltase (7), their association, and their unusual anchoring in the MVM (8, 9) would most readily be explained by the synthesis of a single-chain precursor composed of both sucrase and isomaltase, which subsequently would be split into the mature subunits. It seems rather unlikely in our opinion that sucrase and isomaltase are synthesized as two independent high molecular weight precursors and appear on gels as only one band of radioactivity (as could be expected if the two precursors were of almost identical size) because not more than two cleavage products were found on gels after elastase treatment of P.

The ability of elastase to cleave the putative SI precursor *in vitro* points to a new, possibly important, role of the pancreas for the digestive process of the small intestine *in vivo*. Pancreatic proteases might be responsible for the late posttranslational modification or even activation of intestinal membrane enzymes *in vivo*. To date, however, we do not know if the cleavage of P is indeed the mechanism by which the enzyme complex is activated or if P itself is enzymatically active prior to cleavage.

Finally, the transport of P from the GM to the MVM supports the membrane flow theory for the biogenesis of the plasma membrane (2, 29). The obvious advantage of our system is the effective separation of MVM from GM (12, 13, 15), combined with the absence of cleaved SI in the latter, ruling out the possibility of mutual cross-contamination. Late posttranslational processing on the level of the plasma membrane might in part be responsible for the divergence in protein composition of Golgi and surface membranes in various cell types (3, 12, 30).

Note Added in Proof. We have recently transplanted fetal intestines under the skin of rats according to Leapman *et al.* (31). Under these conditions a normal morphological and biochemical maturation of the isolated gut occurs within 2–3 weeks. When MVM from these transplants were analyzed, using the same antibody and experimental conditions described in this paper, a single protein was found that had both sucrase and isomaltase activity and comigrated with P on sodium dodecyl sulfate/polyacrylamide gels. No processed SI subunits were observed. This suggests that P is enzymatically active and is not split into subunits in the absence of luminal proteases.

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