CELL-FREE SYNTHESIS OF THE ONE-CHAIN PRECURSOR OF A MAJOR INTRINSIC PROTEIN COMPLEX OF THE SMALL-INTESTINAL BRUSH BORDER MEMBRANE (PRO-SUCRASE–ISOMALTASE)

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1. Introduction

The dimeric sucrase–isomaltase complex (SI) (M_r, 275 000 [1]; M_r of the subunits by SDS–PAGE, 120 000 and 140 000, respectively [2]) accounts for ~10% of the intrinsic proteins of the brush border membrane of the small-intestine. The bulk of the protein mass is exposed to the luminal side of the membrane [2]. Its positioning has been elucidated as follows:

(i) The isomaltase (I) subunit is anchored to the membrane via a highly hydrophobic loop located near the N-terminus of the polypeptide chain [2,3]; the segment between residues ~12 and ~60, which includes a Pro at position 35, is nearly totally in an α-helical configuration and presumably crosses the membrane bilayer twice [4].

(ii) No direct interaction of the sucrase (S) subunit with the membrane fabric could be detected [2].

(iii) The N- and C-termini of sucrase and the C-terminus of isomaltase are exposed to the luminal side [2].

(iv) The segment 1–11 of isomaltase is also located at the luminal side (Thr-11 is glycosylated [3,5] and the N-terminus can be labeled by an impermeant reagent [6]).

In order to explain within a single framework this particular arrangement of the SI complex, the homology between the 2 subunits (review [7,8]) and their related or common hormonal control (review [7]), one of us suggested in 1978 the 'one-chain, two-active-sites precursor hypothesis' [9]. According to it, the 2 subunits have arisen from a common ancestor gene (coding for an isomaltase with maltase activity) by partial gene duplication giving rise to one polypeptide chain carrying two identical active sites; subsequent mutation(s) changed the substrate specificity of one of the sites: while maltose would still be accepted and hydrolysed, isomaltose would not be so any more, whereas sucrose would now be hydrolysed. This 'one-chain, two active-sites sucrase–isomaltase' (pro-SI) would be inserted into the membrane during synthesis and then split into the two chains of the 'final' SI complex by extracellular (e.g., pancreatic) proteases.

A one-chain pro-SI in the 260-kDa-range was isolated from small intestines which had not been exposed to pancreatic proteases [10,11], which in all likelihood is identical with the large M_r precursor found to migrate from the Golgi membranes to the brush border in pulse-chase experiments [12]. Pro-SI is indistinguishable from 'final' SI both enzymically and immunologically, which shows that the prospective S and I subunits are preformed in this precursor [10]. The I portion is at the N-terminal segment of pro-SI [5,11]. Treatment of pro-SI with pancreatic elastase leads to bands with apparent M_r-values close to those of the subunits of 'final' SI [10,12].

Many aspects of the mechanism of biosynthesis and membrane insertion of pro-SI remain to be eluci-

Abbreviations: M_r, relative molecular mass; SI, sucrase–isomaltase complex; pro-SI, pro-sucrase–isomaltase, S, sucrase; I, isomaltase; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; kDa, kilodalton

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dated. As a further step towards this goal we have now succeeded in translating one-chain pro-SI polypeptide(s) in a cell-free system. These polypeptides react with antisera raised against native or denatured SI. On SDS–PAGE they show M_r-values in the 270 000 range. Peptide mapping indicates their close similarity to SI.

2. Experimental

Guanidinium thiocyanate was from Fluka; all other chemicals were of analytical grade. [^{35}S]Methionine ('translation grade', spec. act. ~1000 Ci/mmol) was from NEN, micrococcal nuclease and calf liver tRNA were from Boehringer, amino acids from Pierce. Dog pancreas microsomes were prepared according to [13]. Antiserum against rabbit SI was raised in guinea pigs. *Staphylococcus aureus,* strain Cowan I, was fixed with glutaraldehyde and heat-inactivated [14, 15]. Glassware was made nuclease-free by heating overnight (180°C); plasticware was soaked in a 0.2% aqueous solution of diethylpyrocarbonate and autoclaved. All solutions were made with water treated with 0.2% diethylpyrocarbonate followed by autoclaving. Antiserum against denatured SI was raised in a goat using 1 mg antigen in 0.5 ml phosphate-buffered saline mixed with 0.5 ml complete Freund's adjuvant followed by a booster injection (1 mg antigen in 1 ml incomplete Freund's adjuvant) 1 month later. One week before each bleeding, another boost of 200 μg in 1 ml incomplete Freund's adjuvant was given. The antigen was prepared by boiling purified Triton-solubilized SI [16] in 2% SDS, 10% 2-mercaptoethanol. SDS was removed by extensive dialysis against distilled water and against phosphate-buffered saline.

2.1. *Isolation of total RNA*

Total RNA was extracted with 4 M guanidinium thiocyanate from fresh mucosal scrapings of rabbit small intestines and purified by repeated precipitation from 6 M guanidinium–HCl with ethanol [17]. The A_{260}/A_{280} ratios were in the range of 1.8–2.2.

2.2. *In vitro protein synthesis*

The cell-free translation system from rabbit reticulocyte lysate was used. Endogenous mRNA was digested with Ca^{2+}-dependent micrococcal nuclease. Preparation of lysate, conditions for translation, and assay of incorporated radioactivity were as in [18]. Translations were performed in the presence of 0.5–1.5 mCi [^{35}S]methionine/ml lysate, 1.5–3.0 mg total RNA and 70 μg tRNA from calf liver, in the presence or absence of dog pancreas microsomes.

2.3. *Immunoprecipitation*

The in vitro synthesized SI was purified using anti-SI antiserum and *Staphylococcus aureus* [14]. The translation mixture (up to 1 ml) was made 1% in Triton X-100 and diluted 4 times with 50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (pH 7.2) (Triton buffer). A proteinase inhibitor mixture (30 μl) containing 2.8 mg aprotinin/ml, 1 mg/ml each of pepstatin leupeptin, antipain and chymostatin, respectively, was added. After centrifugation at 100 000 × *g* for 1 h, the pellet (100 000 × *g* pellet) was resuspended in a very small volume of water. SDS and 2-mercaptoethanol were added to a final concentration of 3% and 1%, respectively, and the mixture was boiled for 2 min. After centrifugation as above, the supernatant was lyophilized, taken up in Triton buffer and diluted to 0.1% SDS. After another centrifugation, 100 μl goat anti-denatured SI antiserum was added and incubated at 4°C overnight. After addition of 100 μl of a 10% *Staphylococcus aureus* suspension the incubation continued for 2 h. After centrifugation the supernatant was incubated with another 100 μl *Staphylococcus* suspension. The combined pellets were washed 3 times with 0.2 ml Triton buffer containing 0.1% SDS, resuspended in a small volume of water, and heated in 5% SDS, 1% mercaptoethanol. The pellets were re-extracted 3 times with water and the combined supernatants were lyophilized, dissolved in Triton buffer to a final SDS concentration of 0.1%, and immunoprecipitation was repeated with 100 μl goat antiserum. The final staphylococcal pellet was resuspended in 50 μl SDS–PAGE sample buffer and heated for 5 min in boiling water, the *Staphylococcus* cells were re-extracted twice, and the combined supernatants were examined by SDS–PAGE.

For precipitation with the anti-native SI-antiserum from guinea pig 100 μl antiserum were added to the supernatant of the 100 000 × *g* pellet. The treatment with *Staphylococcus* was carried out as above; for the second precipitation goat antiserum was used.

2.4. *SDS–PAGE*

Electrophoresis was performed using a discontinuous sulfate–borate system modified from [19], and

consisting of a (3.2 × 2.6)* polyacrylamide stacking gel buffered in 0.055 M Tris–H_2SO_4 (pH 6.14), 0.1% SDS and a (6.2 × 2.6) separation gel buffered in 0.74 M Tris–HCl (pH 9.18), 0.1% SDS. Cathode (upper) buffer was 0.044 M boric acid–0.065 M Tris (pH 8.5), 0.1% SDS. Anode (lower) buffer was 0.43 M Tris–HCl (pH 9.28).

2.5. *Fluorography*

This was performed as in [21].

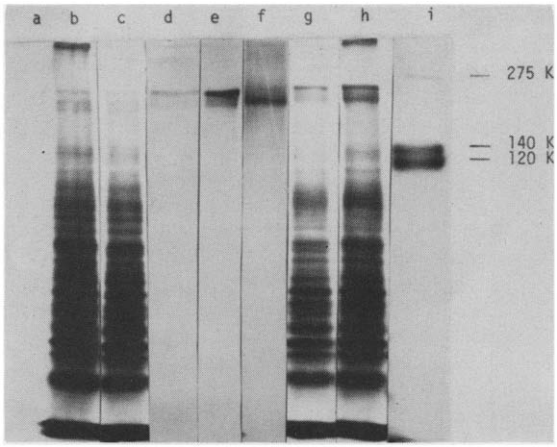

Fig.1. In vitro synthesis of pro-SI (or a precursor thereof). Fluorograph of ^{35}S-labelled polypeptides synthetized in a reticulocyte lysate pretreated with nuclease [18] in the presence or absence of dog pancreas microsomes, in response to total RNA extracted from rabbit intestinal mucosa. After translation, the synthetized polypeptides were precipitated with anti-SI-antiserum (for details, see text). The SDS–polyacrylamide gels (6%) were exposed for fluorography for 4 days (a–d,g,h) or 2 days (e,f): (a) control (no RNA, no microsomes); (b) translation mixture without microsomes; (c) as in (b) centrifugation after incubation (100 000 × *g* supernatant); (d) immunoprecipitate from (c); (e) mixture of the following 3 immunoprecipitates: (d) + immunoprecipitate from the 100 000 × *g* pellet (see (c)) + immunoprecipitate from (g) (see below); (f) translation mixture with microsomes, spun at 100 000 × *g* after the incubation (immunoprecipitate of the pellet); (g) translation mixture with microsomes, spun at 100 000 × *g* after the incubation (supernatant, no immunoprecipitation); (h) translation mixture with microsomes; (i) ^{125}I-SI (120-kDa, sucrase subunit; 140-kDa, isomaltase subunit).

* The first numeral refers to the total weight of monomer (acrylamide + *N,N'*-methylenebisacrylamide, gramme percent, w/v) and the second numeral to the amount of *N,N'*-methylenebisacrylamide, expressed as a percentage (w/w) of the total amount of monomer [20]

2.6. *Peptide maps*

These were obtained by treatment of gel pieces containing the protein with 75% (v/v) formic acid for 40 h at 37°C [22]. The fragments were separated with a SDS–10% PAGE according to [23].

3. Results and discussion

To the best of our knowledge, pro-SI is the largest reported intrinsic membrane polypeptide synthetized in vitro from mRNA (fig.1). The translation system used produced some high-M_r bands, of which two (M_r by SDS–PAGE, 270 000 and 240 000, fig.1(d–f)) were precipitated by anti-SI-antiserum. If pre-immune serum was used instead, no band could be detected in the fluorogram, even after a 6-times longer exposure (not shown).

When translation was carried out in the presence of dog pancreas microsomes (fig.1(h) the immuno-

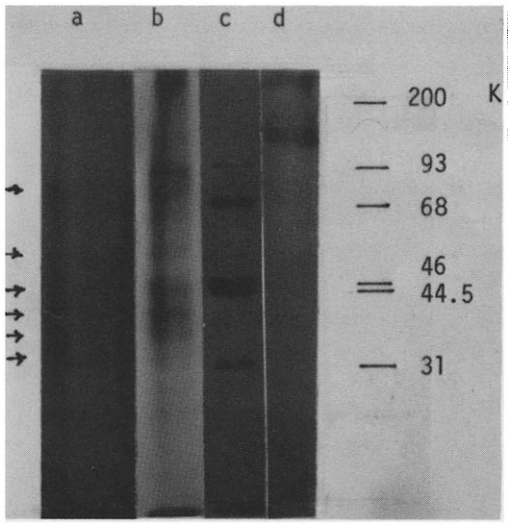

Fig.2. Peptide map of the immunoprecipitated translation product. A gel piece containing the faster moving protein band (as in fig.1(f)) was subjected to treatment with 75% formic acid [22] and re-electrophoresed on an SDS–polyacrylamide (10%) gel according to [23]. Fluorography was for 4 days: (a) peptides from the translation product ([^{35}S]-methionine labelled); (b) peptides from ^{125}I-SI; (c) ^{14}C-labelled standard M_r proteins (myosin, 200-kDa; glycogen phosphorylase, 93-kDa; bovine serum albumin, 68-kDa; cytosolic aspartate aminotransferase, 46-kDa; mitochondrial aspartate aminotransferase, 44.5-kDa; carbonic anhydrase, 31-kDa; (d) ^{125}I-SI. The arrows indicate the peptide bands appearing in both (a) and (b).

specific bands were much more intense than after translation in the absence of microsomes (fig.1(b–d)). In addition, the band with higher mobility was almost lacking in the supernatant of the 100 000 × *g* pellet (fig.1(g)), from which it could be extracted with SDS and specifically precipitated (fig.1(f)). At the moment it cannot be decided whether this indicates the processing of a (still hypothetical) pre-pro-SI or whether the higher-mobility band arises from the other one by a proteolysis unrelated to the processes of translation and membrane insertion. Interpretation of this mobility change is complicated, at this stage, by the possibility that these polypeptides might be glycosylated, the sugar moieties of rabbit SI accounting for ~15% of its weight [24,25].

The similarity in the peptide patterns of ^{125}I-SI and of the [^{35}S]Met-labelled in vitro translation product (fig.2) confirms that the one-chain large-M_r translation product(s) is (are) indeed identical with, or a precursor of, pro-SI.

Our data, therefore, further show that SI is synthetized as a large one-chain precursor and rule out the possibility, indicated by others, that S and I may instead be synthetized independently.

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