Intracellular degradation and reduced cell-surface expression of sucrase–isomaltase in heat-shocked Caco-2 cells

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To investigate the role of post-translational events in intestinal cell differentiation we have studied the effects of heat shock on processing and cell surface delivery of sucrase–isomaltase (SI), dipeptidylpeptidase IV (DPPIV) and aminopeptidase N (APN) in Caco-2 cells. In cells cultured at 42.5 °C there was a rapid decline in sucrase activity, while DPPIV and APN were unaffected over a 3-day period. Immunofluorescence staining confirmed the selective disappearance of SI from the surface membrane after only 1 day of culture at 42.5 °C. Cell-surface biotinylation of cells metabolically labelled with [^{35}S]methionine 4 h after a switch from 37 °C to 42.5 °C demonstrated that newly synthesized APN and DPPIV were associated with the surface membrane, while SI was almost completely retained intracellularly. Pulse–chase experiments confirmed that, in these cells, DPPIV and APN were normally processed and vectorially delivered to the cell surface; in contrast, conversion between the two conformationally distinct high-mannose precursor forms of SI (hmP1 and hmP2) was markedly inhibited, a significant fraction of newly synthesized enzyme was degraded, probably in the ER, and an immature form of complex-glycosylated SI precursor (cP) was produced and mostly retained intracellularly. Double labelling of Caco-2 cells for SI and cathepsin D excluded an accumulation of SI in the lysosomes, suggesting that this organelle was not involved in the degradation of SI. These results indicate that the ER may play an important role in intestinal cell differentiation by regulating the conformational maturation, degradation and eventual cellular localization of some digestive enzymes.

INTRODUCTION

A form of sucrase–isomaltase (SI) that is immunologically and conformationally distinct from that expressed by mature enterocytes has been detected in proliferative small intestinal [1,2] and colonic [3] crypt cells, suggesting that post-translational processing of this enzyme may differ as a function of cell differentiation. Alterations in the intracellular sorting, degradation and cell-surface delivery of brush-border enzymes have also been implicated in the regulation of lactase and SI expression in newborn and adult animals, and in several cases of congenital SI deficiency [4–6].

SI is a marker enzyme that is particularly well suited to the study of intestinal cell differentiation and its regulation, because much is known regarding its structure and biosynthesis [7,8]. It is a Type II integral membrane protein that is localized in the luminal (brush-border) membrane of differentiated enterocytes and is synthesized as a single-chain precursor which, *in vivo* [9] but not in cultured intestinal cell lines [1,10–12], is split into the sucrase and isomaltase subunits by extracellular proteases. At least two distinct intracellular high-mannose precursors have been identified [1,10]: the initial translation product (hmP1), which is very sensitive to proteolytic degradation [13], and an immunologically and conformationally distinct derivative (hmP2), formed within 1 h of synthesis [while still in the endoplasmic reticulum (ER)] [1,10]. A third form of high-mannose precursor has been identified and localized in the intermediate compartment or in the Golgi complex [13].

Evidence has been obtained suggesting that SI expression *in vivo* and in cultured human colon tumour cell lines is controlled, at least in part, at the post-translational level. In HT-29 cells cultured in the presence of glucose (undifferentiated state), SI synthesis is followed by delivery to a leupeptin-sensitive degradative compartment [14], while in the same cells deprived of glucose (differentiated), the enzyme is properly inserted into the luminal surface membrane [12,14]. In Caco-2 cells, physiological levels of epidermal growth factor had no significant effects on SI mRNA abundance or SI synthesis, but depressed markedly sucrase activity by influencing the conversion between the hmP1 and hmP2 forms of SI, with a concomitant increase in its intracellular degradation [15]. Analysis of Caco-2 clones endowed with markedly different sucrase activities and levels of endogenous growth factors gave similar results, suggesting that both transcriptional and post-translational mechanisms act synergistically to regulate SI expression in Caco-2 cells [16]. In these studies the activities of other brush-border enzymes, such as aminopeptidase N (APN), dipeptidylpeptidase IV (DPPIV) and alkaline phosphatase, showed much less variability than that of SI, indicating that the overall programme of Caco-2 cell differentiation was not affected. Hauri and his co-workers have demonstrated that brush-border enzyme glycoproteins are transported to the cell surface at markedly different rates (APN and

Abbreviations used: APN, aminopeptidase N; cP, mature sucrase–isomaltase precursor, with complex glycosidic chains; DME, Dulbecco's modified Eagle medium with 4.5 g/l glucose; DPPIV, dipeptidylpeptidase IV; DTT, dithiothreitol; endo-H and -F, endo-β-*N*-acetylglucosaminidases H and F respectively; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; FCS, fetal calf serum; hmP1, newly synthesized sucrase–isomaltase precursor, with glycosidic chains of the high-mannose type; hmP2, sucrase–isomaltase high-mannose precursor 2; hsp, heat shock protein; PMSF, phenylmethanesulphonyl fluoride; SI, sucrase–isomaltase; S-NHS-biotin, sulpho-*N*-hydroxysuccinimido-biotin; S-NHS-SS-biotin, sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate.

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DPPIV much more rapidly than SI or lactase), and suggested that this asynchrony is due to at least two rate-limiting events: a pre- and an intra-Golgi step [10,17,18]. These results mentioned above point to an interesting correlation between intracellular degradation or mis-sorting of brush-border enzymes and their rates of exit from the ER, suggesting that this organelle may play an important role in intestinal cell differentiation. It is well established that ER-resident proteins contribute to the proper folding of newly synthesized secretory and membrane proteins [19–26], and that proteins which fail to undergo required conformational changes are retained in the ER and eventually selectively destroyed there by one or more specific degradation systems [27–29].

In this study we have used heat shock to investigate the role of the ER in the expression and intracellular processing of brush-border enzymes in Caco-2 cells. The results obtained demonstrated that, in heat-shocked cells, the predominant form of SI synthesized is immunologically related to that produced by proliferative crypt cells *in vivo* [1] and is very inefficiently transported to the cell surface, suggesting that intestinal epithelial cell differentiation may be, at least in part, centred on changes in ER-related functions.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DME) and irradiated fetal calf serum (FCS) were from Whittaker Bioproducts (Walkersville, MD, U.S.A.). Triton X-100, endo-β-*N*-acetylglucosaminidase H (endo-H), endo-β-*N*-acetylglucosaminidase F (endo-F), methylated ^{14}C-labelled molecular mass markers (carbonic anhydrase, BSA, phosphorylase B, globulins and myosin), and L-[^{35}S]methionine (1083–1151 Ci/mmol) were obtained from Dupont–NEN (Boston, MA, U.S.A.); Tris, phenylmethanesulphonyl fluoride (PMSF), aprotinin, leupeptin and antipain were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sulpho-*N*-hydroxysuccinimido-biotin (S-NHS-biotin), sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (S-NHS-SS-biotin) and streptavidin–agarose beads were from Pierce Chemical Co. (Rockford, IL, U.S.A.); Affigel 10 was from Bio-Rad (Melville, NY, U.S.A.).

Antibodies

The following mouse monoclonal antibodies, prepared and characterized as described elsewhere, were used in this study: HSI-14 [1], specific for the isomaltase subunit and recognizing all forms of SI (both native and denatured), including the initial high-mannose SI precursor (hmP1) and the SI expressed by undifferentiated crypt cells *in vivo* [1,3]; HSI-5 [1], specific for a conformation-dependent epitope associated with hmP2 (but not hmP1) and for the enzymically active mature forms of SI expressed by absorptive villus cells *in vivo* and by Caco-2 cells; HBB2/45 [30], specific for human APN; and DAO7/219 [31], specific for human DPPIV. All antibodies mentioned above are of the IgG_1 subtype and were used as hybridoma-conditioned media or as immunoglobulins purified from ascites fluids [1]. For use in immunoprecipitations they were coupled to Affigel-10 beads following the instructions of the manufacturer (Bio-Rad). A rabbit antiserum prepared against bovine cathepsin D was generously provided by Dr. William Brown (Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY, U.S.A.).

Cell culture and experimental treatments

The human colon tumour cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Clones expressing different amounts of SI were obtained by dilution plating and were characterized as described elsewhere [16]. Clone #15 (Caco-2/15 cells), endowed with the highest sucrase specific activity (expressed as units/mg of total cell protein), was selected for the studies described in this paper, and was routinely grown and subcultured as described [1,16]. Cells to be used for metabolic labelling with [^{35}S]methionine were seeded at a density of 8×10^6 cells per filter in Transwell clusters, 24.5 mm in diameter (surface area 4.71 cm^2), obtained from Costar (Cambridge, MA, U.S.A.); 2.5 ml of medium per well was added to the cluster plates (lower chamber volume) and 1.5 ml medium was added to the Transwells (upper chamber volume). The cells were used between 14 and 21 days after seeding to ensure optimal polarization of the cells and maximal expression of SI, DPPIV and APN.

Heat-shocked cells were placed at 42.5 °C for 1–3 days (for determination of enzyme activities and immunofluorescence studies), or for 4 h before labelling with [^{35}S]methionine (also done at 42.5 °C). Control cells were maintained at 37 °C, the normal growth temperature.

Labelling of Caco-2/15 cells with radioactive methionine

Cells on Transwell filters were pre-incubated twice with methionine-free medium (for 20 min each time) to deplete intracellular methionine pools, and then with fresh medium containing 250–500 μCi/ml L-[^{35}S]methionine (1034–1176 Ci/mmol) as previously described [15]. Both steps were performed at 42.5 °C for heat-shocked cells. Labelling (pulse) periods were 15 min to 1 h (pulse–chase experiments) or 1–6 h (other metabolic labelling experiments). In pulse–chase experiments, after incubation with radioactive methionine the cells were rinsed twice with standard complete medium and further incubated with complete medium supplemented with 5 mM unlabelled methionine.

Cell-surface biotinylation, immunoprecipitation and analysis of labelled proteins

Cells cultured on Transwell filters were biotinylated from the apical or basolateral side (or both sides at the same time), and then processed for immunoprecipitation and streptavidin precipitation according to Le Bivic et al. [30]. Total cell membrane fractions were obtained, solubilized and immunoprecipitated with antibodies bound to Affigel-10 beads as described [15]. All buffers also contained the same cocktail of protease inhibitors used for cell homogenization, membrane purification, solubilization and immunoprecipitation [15].

Antigens were eluted [32] from the antibody–bead conjugates and analysed by SDS/PAGE under reducing conditions [50 mM dithiothreitol (DTT)]; SDS/PAGE and detection of labelled proteins by fluorography were performed as previously described [33] using 6% or 7.5% acrylamide gels. Gels impregnated with EN^{3}HANCE (Dupont–NEN) were dried and exposed to XAR-5 films for 1–60 days; multiple exposures of the same gel for different lengths of time were obtained in all cases. The intensity of the bands on the fluorographs was assessed, within the limits of linearity, by using a laser densitometer (Ultroscan XL; Pharmacia LKB, Piscataway, NJ, U.S.A.). Alternatively, the radioactivity associated with individual proteins, excised from the gels and solubilized as described by Stieger et al. [18], was determined using a Beckman LS 3800 liquid scintillation counter.

Glycosidase treatments

Washed immunoprecipitates (consisting of labelled antigens bound to Affigel-10-linked antibodies) were processed and digested with endo-H or endo-F, according to Matter et al. [17]. In some experiments, parallel samples were digested with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (*N*-glycanase) as follows. The immunoprecipitates, after washing with 0.2 M sodium phosphate, pH 8.2, were boiled for 5 min in 20 μl of 0.5% SDS, 50 mM 2-mercaptoethanol and 0.2 M sodium phosphate, pH 8.2. After cooling to room temperature, to the samples was added 76 μl of 0.1 M sodium phosphate, pH 8.2, 2% Nonidet P40, containing a mixture of protease inhibitors (1 μg/ml antipain, 17.5 μg/ml benzamidine, 1 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM PMSF), and finally 0.625 units (2.5 μl) of *N*-Glycanase; digestions were for 18 h at 37 °C. The reactions were stopped by adding 100 μl of 2 × concentrated SDS/PAGE sample buffer followed by incubation at 60 °C for 5 min. The beads were separated by centrifugation and the supernatants were analysed by SDS/PAGE.

Immunofluorescence staining

Post-confluent Caco-2 cells, grown in 35 mm dishes at 37 °C or 42.5 °C as indicated, were rinsed with PBS, and then fixed and stained by either of two methods.

(a) For visualization of cell-surface-associated SI, DPPIV or APN, cells were incubated with 3% paraformaldehyde in PBS, pH 8.3, at room temperature for 20 min with shaking. Thereafter, the cells were washed twice with PBS, incubated with 100 mM glycine in PBS at room temperature for 30 min, washed twice more with PBS, incubated with the first antibody (ascites fluids, diluted 1:200 in PBS; 0.5 ml/dish) for 30 min, washed three times with 2 ml of PBS (10 min each time), incubated with the second antibody [fluorescein isothiocyanate (FITC)-conjugated goat anti-(mouse Ig), $F(ab')_2$ fragment, from Boehringer-Mannheim; diluted 1:25 in PBS; 0.5 ml/dish] for 30 min and washed three times with 2 ml of PBS (10 min each time). Cells were counterstained with 0.1% Evans Blue for 30 s, then again washed with PBS.

(b) For double labelling with antibodies to SI and cathepsin D, cells were incubated with 3% paraformaldehyde in PBS, pH 8.3, at room temperature for 20 min, washed twice with PBS, incubated with 100 mM glycine in PBS for 10 min, incubated with 0.1% saponin in 100 mM glycine in PBS for 20 min, and incubated for 15 min with 0.1% saponin in PBS + 2% BSA. The following steps were as in (a), except that 0.1% saponin was added to all solutions containing primary and secondary antibodies, and to the PBS used for washing. The second antibody mixture consisted of FITC-conjugated goat anti-(mouse Ig), $F(ab')_2$ fragment, and rhodamine-conjugated sheep anti-(rabbit IgG), both diluted 1:25 in PBS. Non-immune mouse or rabbit sera were used as negative controls. Cells were mounted with glycerol/PBS (9:1, v/v) containing 2.5% 1,4-diazabicyclo-[2.2.2]octane. Stained cells were examined with a Zeiss Axiovert 10 microscope coupled with a Bio-Rad MRC-600 confocal laser scanning attachment; fluorescent images were saved to an optical disk and subsequently transferred to a Kodak Plus-X pan 125 B/W film.

Enzyme assays

A crude total membrane fraction was obtained from cells grown in 100 mm dishes as previously described [16]. Proteins were determined by the method of Lowry et al. [34]. Sucrase was assayed by the method of Messier and Dahlqvist [35]; APN and DPPIV were assayed according to Roncari and Zuber [36], with L-leucine *p*-nitroanilide or glycyl-L-proline *p*-nitroanilide *p*-tosylate respectively as substrates.

RESULTS

Brush-border enzyme expression in Caco-2 cells cultured at 37 °C and 42.5 °C

Levels of sucrase, DPPIV and APN activities were assessed in total membrane fractions isolated from Caco-2/15 cells maintained at the normal growth temperature (37 °C, controls) or shifted to 42.5 °C for 1–3 days. The cells were 14 days confluent at the beginning of these experiments; thus there was very limited cell proliferation during the test period, and total protein values did not differ by more than 20% among the samples examined.

As shown in Figure 1, only sucrase was markedly affected by the shift in temperature: after only 1 day at 42.5 °C its specific activity (units/g of protein) was 50% lower than in cells kept at 37 °C, and by the third day this had further declined to 18% of the control value. In contrast, in the same cultures DPPIV specific activity was not significantly different in cells maintained at 37 °C and 42.5 °C, and APN showed only a slight decrease

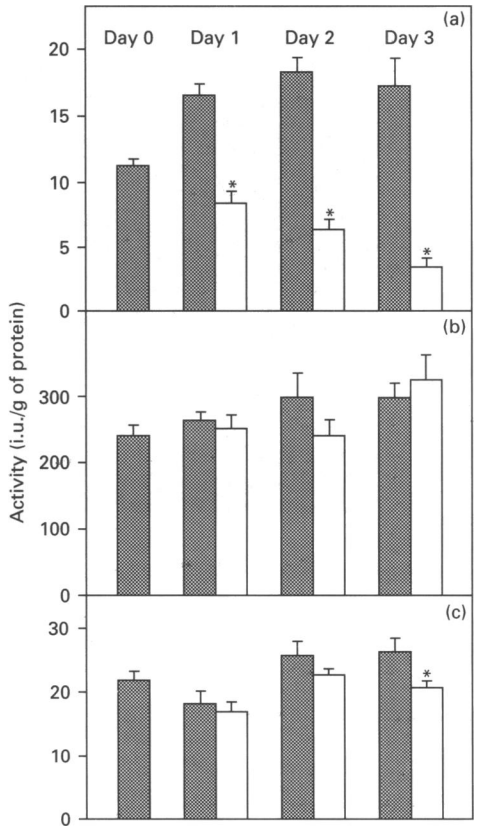

Figure 1 Enzyme activities associated with Caco-2/15 cells confluent for 14 days, grown in 100 mm dishes at 37 °C, and then either kept at that temperature (▨) or shifted to 42.5 °C (□) for 1–3 days

Total membrane fractions were prepared and tested for sucrase (**a**), DPPIV (**b**) and APN (**c**) activities as described in the Materials and methods section. Each bar represents the mean ± S.E.M. of three samples. *Statistically significant difference ($P < 0.05$).

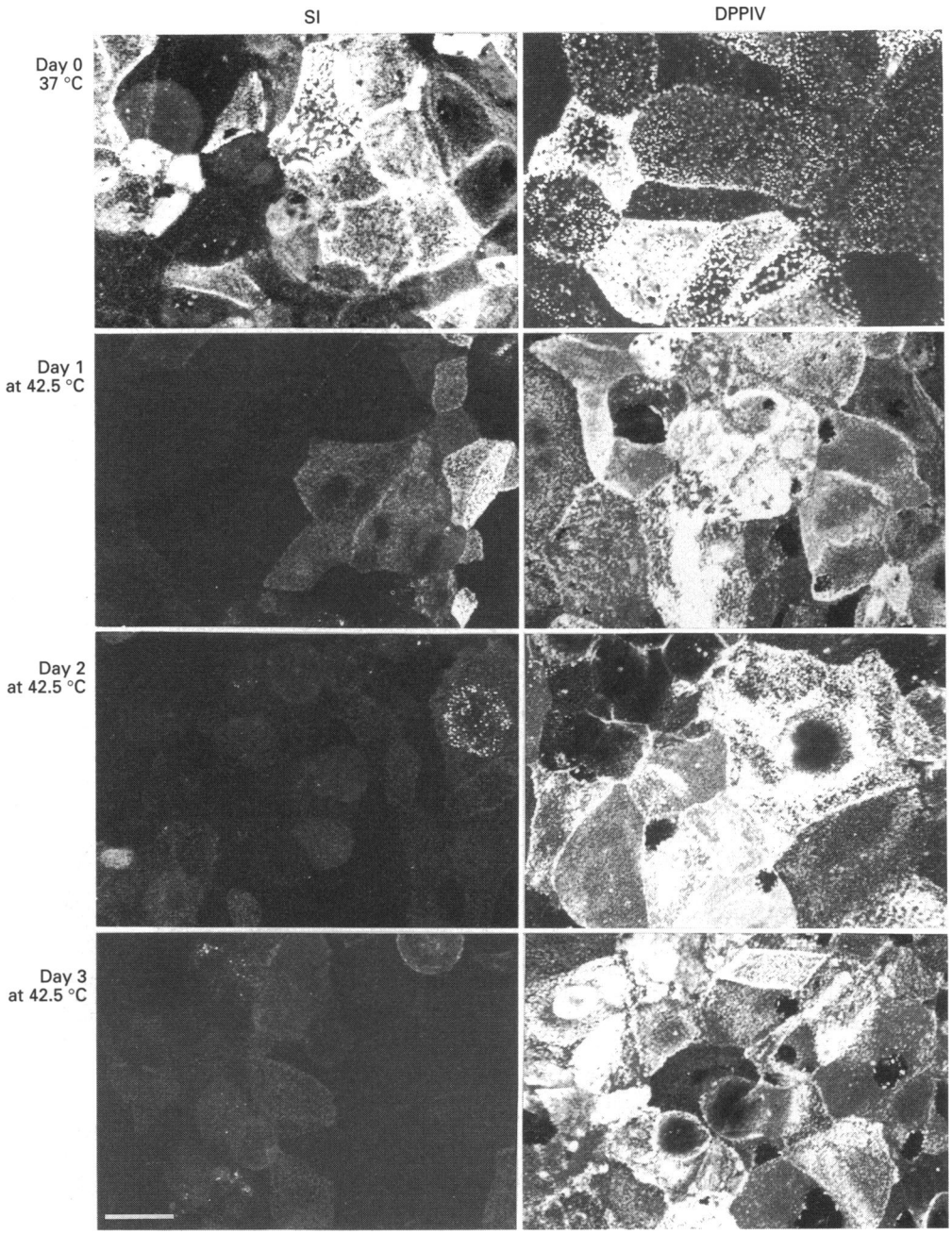

Figure 2 Indirect immunofluorescence staining of Caco-2/15 cells with the monoclonal antibodies HSI-14 and DA07/219, specific for SI and DPPIV respectively

Cells confluent for 10 days, grown in 35 mm dishes at 37 °C, were either fixed and stained or shifted to 42.5 °C on day 0; thereafter, cells cultured at 42.5 °C were examined at daily intervals. In all cases, cells were fixed with formaldehyde (cell surface staining procedure) and stained as described in the Materials and methods section. Bar = 25 μm.

that reached statistical significance after 3 days of culture at 42.5 °C.

Immunofluorescence staining of parallel cultures confirmed and extended these results. The brush-border membrane of the entire cell population was intensely stained for DPPIV (Figure 2) and APN (results not shown) at all times examined, irrespective of the culture temperature. In contrast, most cells were positive for SI when cultured at 37 °C, but after 1 day at 42.5 °C only a few stained cells could be detected, and at later times all cells were either negative or only faintly stained with antibodies to SI (Figure 2). Double labelling of the same cells for SI and DPPIV produced comparable results, and permeabilization with saponin or Triton X-100 did not result in any significant intracellular accumulation of SI at either temperature (results not shown).

Overall, these results demonstrated that culture of Caco-2/15 cells at 42.5 °C did not affect cell viability and morphology, at least at the level of the light microscope, and that SI expression was selectively inhibited.

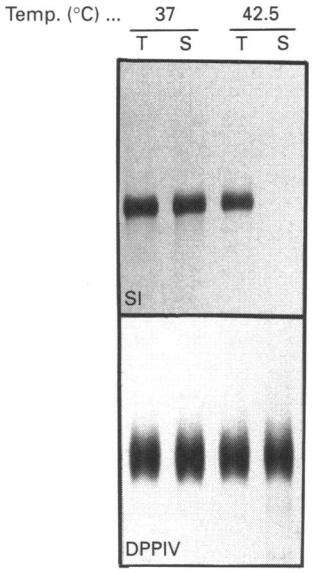

Figure 3 Cell-surface association of SI and DPPIV synthesized by confluent Caco-2/15 cells cultured at 37 °C or at 42.5 °C

Cells, grown on filters, were pulse-labelled with [^{35}S]methionine for 1 h and then chased for 5 h in medium containing 5 mM unlabelled methionine. After pulse labelling, both the apical and basolateral membranes were biotinylated with S-NHS-SS-biotin. SI and DPPIV were sequentially immunoprecipitated from detergent-solubilized total cell membrane fractions with antibodies HSI-14 and DAO7/219 respectively, and subsequently eluted from the immunobeads. Equal aliquots of each eluate were either applied directly to the gel (T samples), or loaded on avidin–agarose beads from which bound (biotinylated) antigens were eluted and also applied to the gel (S samples). SDS/PAGE was on a 6% acrylamide gel under reducing conditions (50 mM DTT); labelled proteins were visualized by fluorography.

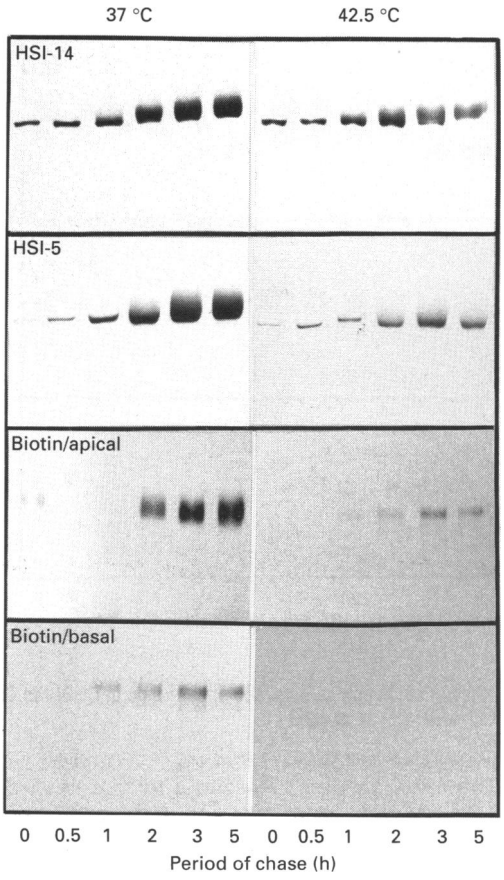

Figure 4 Biosynthesis and cell-surface appearance of SI in Caco-2/15 cells cultured at 37 °C or at 42.5 °C

Cells grown on filters were pulse-labelled with [^{35}S]methionine for 30 min and then chased for the indicated times in medium containing 5 mM unlabelled methionine. Upper two panels: SI was immunoprecipitated from detergent-solubilized total cell membrane fractions with either antibody HSI-14 (recognizing all forms of SI) or antibody HSI-5 (specific for the hmP2 and cP forms of SI). Lower two panels: after pulse-labelling, either the apical or basolateral membranes were biotinylated with S-NHS-SS-biotin; SI was immunoprecipitated from detergent-solubilized total cell membrane fractions with antibody HSI-14, eluted from the immunobeads, and then loaded on avidin–agarose beads; bound biotinylated antigen was eluted and analysed. In all cases antigens were analysed by SDS/PAGE on a 6% acrylamide gel under reducing conditions (50 mM DTT) and visualized by fluorography.

Association of newly synthesized brush-border enzymes with the cell surface

To determine whether SI and DPPIV were correctly delivered to the plasma membrane or mis-sorted in cells cultured at 42.5 °C, we subjected surface membrane proteins to biotinylation [30,37], followed by immunoprecipitation and binding to streptavidin. Confluent Caco-2/15 cells grown on Transwell filters were pulsed for 1 h with radioactive methionine and then chased for 5 h, a period sufficient for nearly complete delivery to the cell surface of all brush-border enzymes in control cells [10,30,37]. Cell-surface biotinylation was performed as described [30] on both the apical and the basal sides of the cells. After immunoprecipitation from cell lysates, SI and DPPIV were released from the beads. Each sample was then divided into two equal aliquots: one (representing the total amount of labelled enzymes synthesized during the pulse period) was applied directly to the gels (samples labelled 'T' in Figure 3), and the other was incubated with streptavidin–agarose beads. Biotinylated (surface membrane-associated) enzymes (labelled 'S' in Figure 3) were then released from the beads and also analysed by SDS/PAGE.

In control (37 °C) cells, most of the SI and DPPIV were found to be associated with the surface membrane. In contrast, in heat-shocked cells, only DPPIV was accessible to cell-surface biotinylation; at most, 10% of the total labelled SI present was bound to streptavidin (a faint band was observed in the autoradiograph shown in Figure 3, which however did not reproduce photographically). These results thus indicated that, in cells grown at 42.5 °C, SI and DPPIV were differentially processed: the former was largely retained in an intracellular compartment, while the latter was delivered correctly to the surface membrane.

Effects of heat shock on intracellular processing, transport and vectorial cell surface delivery of SI and DPPIV

To define the alterations in the intracellular processing of SI in cells cultured at 42.5 °C, we performed a series of pulse–chase experiments, combined with determination of the endo-H sensitivity of the enzymes and domain-specific cell-surface biotinylation [30,37]. In these experiments, confluent Caco-2/15 cells grown on Transwell filters were pulsed for 30 min with radioactive methionine, and then chased for 0–300 min with 5 mM unlabelled methionine. At each time point, cells were either directly used for sequential immunoprecipitation of SI [using either antibody HSI-14, recognizing all forms of the enzyme, or HSI-5, specific for the hmP2 and cP (mature precursor with complex glycosidic chains) forms of SI] and DPPIV, or subjected first to domain-specific cell-surface biotinylation. Fol-

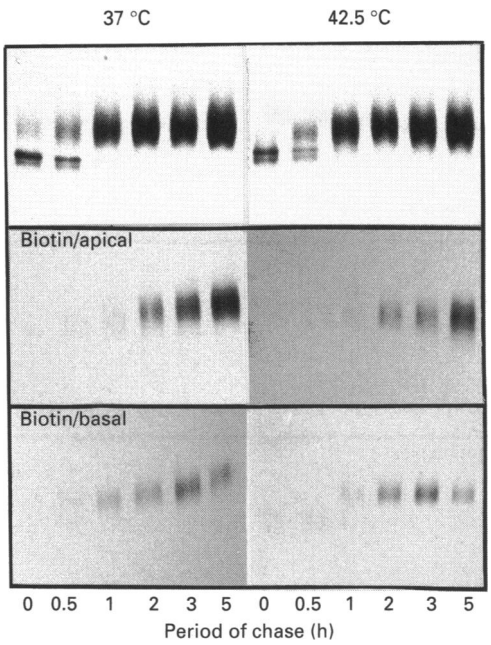

Figure 5 Biosynthesis and cell-surface appearance of DPPIV in Caco-2/15 cells cultured at 37 °C or at 42.5 °C

Cells grown on filters were pulse-labelled with [^{35}S]methionine for 30 min and then chased for the indicated times in medium containing 5 mM unlabelled methionine. Top panel: DPPIV was immunoprecipitated from detergent-solubilized total cell membrane fractions with antibody DA07/219. Lower two panels: after pulse labelling, either the apical or basolateral membranes were biotinylated with S-NHS-SS-biotin; DPPIV was immunoprecipitated from detergent-solubilized total cell membrane fractions with antibody DA07/219, eluted from the immunobeads, and then loaded on avidin–agarose beads; bound biotinylated antigen was eluted and analysed. In all cases antigens were analysed by SDS/PAGE on a 6% acrylamide gel under reducing conditions (50 mM DTT) and visualized by fluorography.

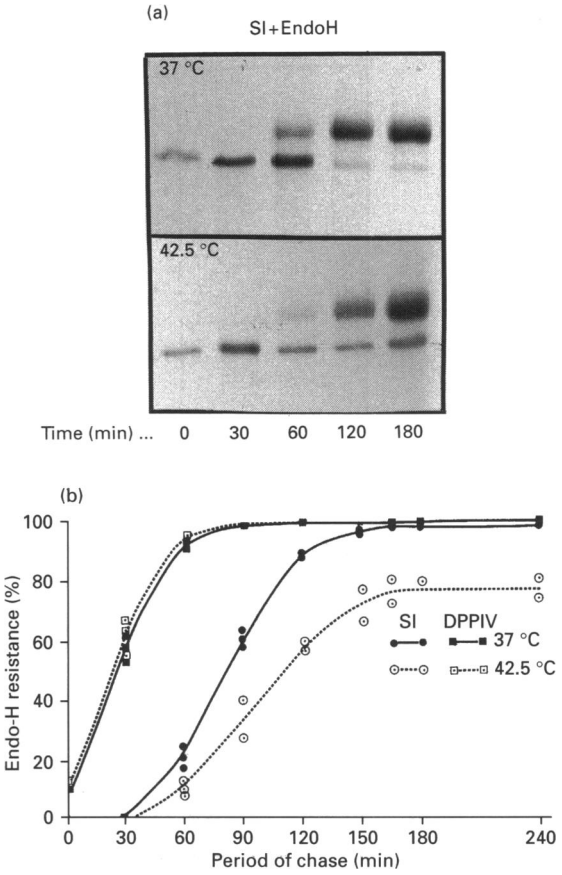

Figure 6 Time course of acquisition of endo-H resistance by SI and DPPIV synthesized by Caco-2/15 cells cultured at 37 °C or at 42.5 °C

Cells grown on filters were pulse-labelled with [^{35}S]methionine for 30 min and then chased for the indicated times in medium containing 5 mM unlabelled methionine. SI and DPPIV, sequentially immunoprecipitated from detergent-solubilized total cell membrane fractions, were digested with endo-H as described in the Materials and methods section, separated by SDS/PAGE on a 6% acrylamide gel under reducing conditions (50 mM DTT), and then visualized by fluorography; representative results obtained for SI in one experiment are presented in (**a**). (**b**) Quantitative evaluation of endo-H resistance from three separate experiments performed as described above.

lowing immunoprecipitation, biotinylated labelled enzymes were separated by incubation with streptavidin–agarose beads. Representative results obtained are presented in Figures 4–6. Overall, three to four sets of similar experiments were performed and quantified by densitometric scanning of the autoradiographs; the cumulative findings are summarized in Figure 7.

Control cultures

Processing and polarized cell-surface delivery of SI and DPPIV followed essentially the same patterns and kinetics previously reported [1,10,13,15,18,30,31,37,38]. In the case of SI, the initial high-mannose precursor hmP1 reached a peak at 30 min of chase and disappeared after 1 h. The half-time for the conversion of hmP1 to hmP2 was 48 min; the derivative hmP2 reached a peak at 60–90 min. Endo-H resistance was acquired with a half-time of 90 min, and by 3 h of chase only traces of hmP were observed, with cP representing at least 95% of the total SI (see Figure 6).

As expected [10,18], DPPIV was processed much faster than SI, acquired endo-H resistance with a half-time of 32 min, and by 1 h of chase had been nearly completely converted into the complex glycosylated form (Figure 6).

SI and DPPIV were inserted initially into both apical and basolateral cell-surface domains, although in very different relative proportions (see Figures 4 and 5). The basal SI reached a peak at 3 h (Figures 4 and 7) and declined thereafter, in accordance with previous studies which demonstrated its rapid transcytosis into the luminal aspect of the cells [30,37].

Heat-shocked cells

Culture of cells at 42.5 °C had marked effects on SI processing and on its final fate. (i) Conversion between hmP1 and hmP2 was dramatically slowed down (the half-time of conversion was 140 min, approx. 3 times more than in controls) (Figures 4 and 7). (ii) The rate of acquisition of endo-H resistance was also considerably slower than in controls (see Figure 6), and a significant proportion of SI (approx. 20%) was still sensitive to endo-H digestion even after 6 h of chase. (iii) The maximal amount of total immunoprecipitable SI present in each pulse–chase experiment was observed at 2–3 h of chase; thereafter, there was a marked decline, suggesting that up to 50% of the newly synthesized SI was degraded within 5–6 h of labelling (see Figures 4 and 7). Addition of leupeptin (100–250 μg/ml) or antipain (100 μg/ml) to the culture medium did not prevent this decline in total labelled SI (results not shown). (iv) Although a significant fraction (40–50%) of newly synthesized SI was converted into cP, most of it failed to react with antibody HSI-5 (see Figure 4). Endo-H digestion of SI immunoprecipitated with antibody HSI-5 after 5 h of chase showed that 70–80% was

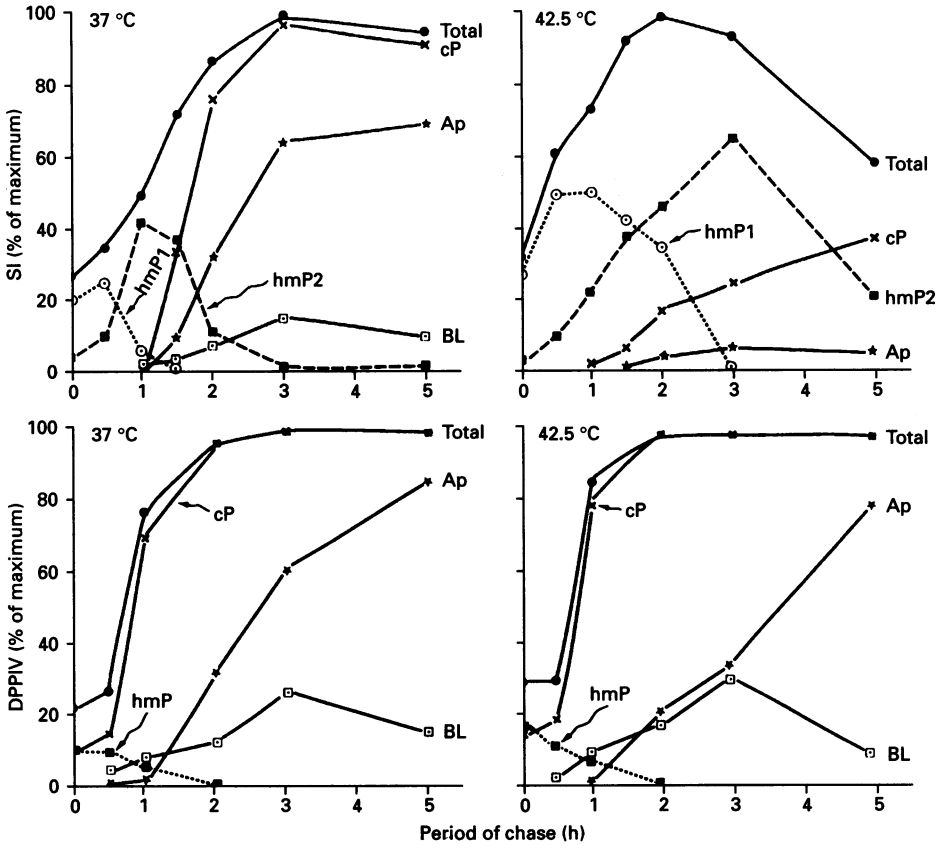

Figure 7 Time courses of conversion among the different biosynthetic forms of SI and DPPIV, and of their appearance at the apical (Ap) or basolateral (BL) surface membranes in Caco-2/15 cells cultured at 37 °C or at 42.5 °C

This Figure presents the summary of a series of experiments performed as described in the legends to Figures 4–6. The relative amounts of hmP and cP present were determined after endo-H digestion of the samples; the relative amounts of SI hmP1 present were calculated by subtracting the amount of hmP immunoprecipitated with antibody HSI-5 (specific for the hmP2 and cP forms of SI) from the amount of hmP immunoprecipitated with antibody HSI-14 from equal aliquots of each cell lysate. The relative amounts of SI and DPPIV present at the apical or basolateral cell-surface membranes were determined by dividing the amounts of biotinylated immunoprecipitated enzymes by the total amounts of immunoprecipitable SI or DPPIV present in the same cells. For each time point, the amounts of hmP, hmP1, hmP2 and cP present are expressed as percentages of the maximal total amount of immunoprecipitable SI or DPPIV obtained in each individual pulse–chase. Each point represents the average of the results obtained in three or four separate experiments.

in the form of hmP2 (results not shown). (v) Only a small fraction (5–10 %) of newly synthesized SI appeared at the apical membrane; none was detected at the basolateral membrane at any time point (Figure 4).

Culture of cells at 42.5 °C did not significantly alter the kinetics of acquisition of endo-H resistance (Figure 6), and of polarized transport to the cell surface, of DPPIV (Figure 5) or APN (results not shown).

SI in heat-shocked cells does not co-localize with lysosomes

Immunoelectron-microscopic studies have revealed a significant accumulation of brush-border enzymes, including SI, in the lysosomes of normal intestinal and Caco-2 cells [39,40]. This finding was not related to endocytosis of proteins previously delivered to the brush-border membrane [40], suggesting that lysosomes may represent a site of intracellular degradation or storage of the enzymes. In view of the rapid disappearance of a significant fraction of newly synthesized SI in Caco-2/15 cells cultured at 42.5 °C, we speculated that this might have been due to increased mis-sorting to the lysosomes. Double labelling of Caco-2/15 cells cultured at 37 °C and 42.5 °C for 1–3 days with antibodies to SI and the lysosomal enzyme cathepsin D seemed to exclude this possibility: although lysosomes were significantly increased in number in cells cultured at 42.5 °C (Figure 8), there was no evidence for an accumulation of SI in this organelle at either culture temperature (Figure 8). Similar results were obtained in double-labelling experiments employing antibodies to DPPIV or APN, and to cathepsin D (results not shown).

DISCUSSION

In the experiments described in this paper, we have used temperature as a probe to investigate the role of structural transitions and intracellular organelles in the regulation of the biosynthesis and expression of brush-border enzymes in intestinal cells. There are many precedents to this approach; for example, lowering the temperature of cells to 16 °C has been used to block the movement of newly synthesized proteins out of the ER [41], and cultured cells subjected to a mild heat stress have been altered in many important physiological properties, leading to their ability to survive otherwise lethal conditions [42]. Among the factors implicated in these processes are all major classes of heat shock proteins (hsps) [42], and organelles such as the ER and mitochondria.

We were particularly interested in probing the role of

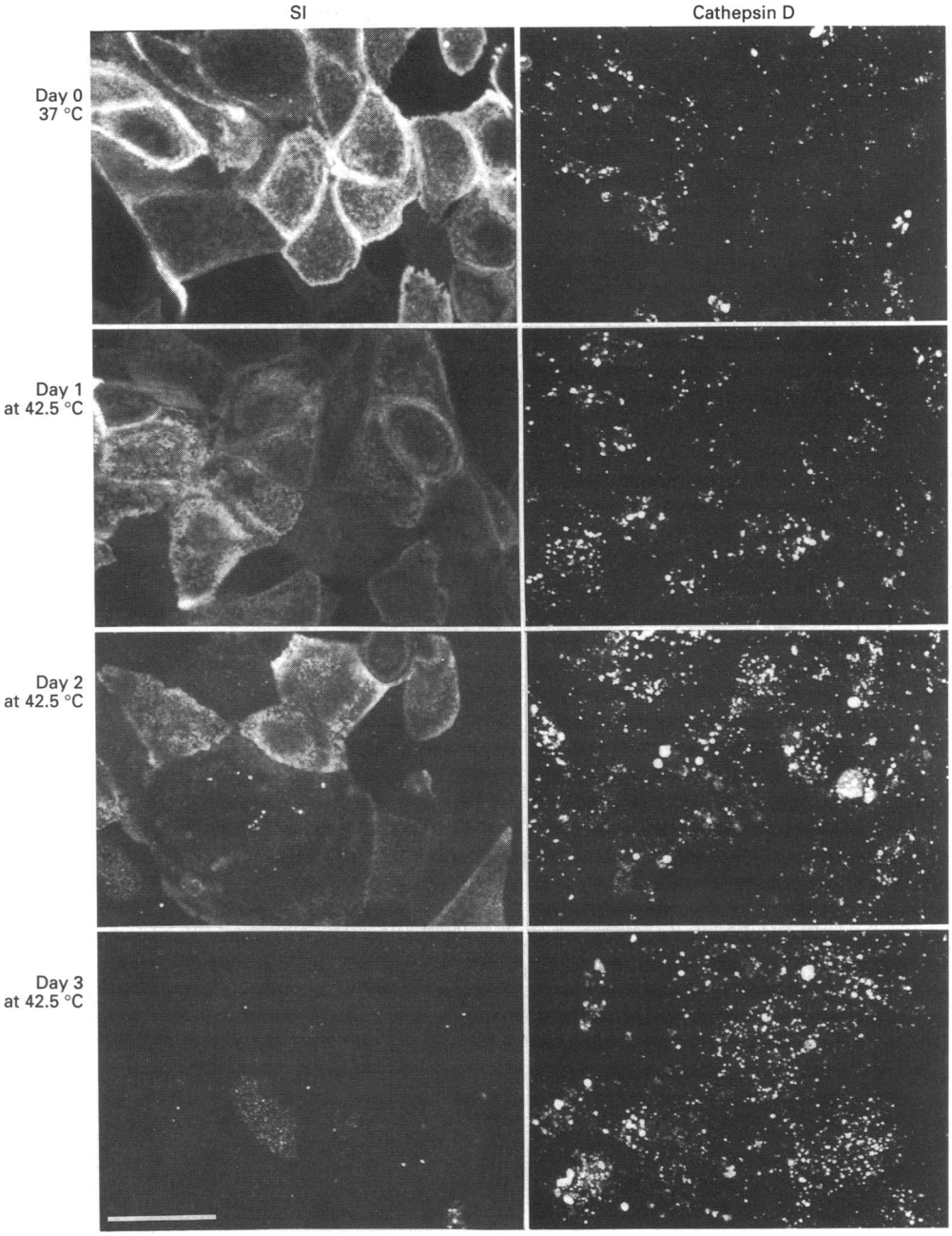

Figure 8 Double labelling (indirect immunofluorescence) of Caco-2/15 cells for SI and cathepsin D

Cells confluent for 12 days, grown in 35 mm dishes at 37 °C, were either fixed and stained or shifted to 42.5 °C on day 0; thereafter, cells cultured at 42.5 °C were examined at daily intervals. In all cases, cells were fixed with formaldehyde, permeabilized with saponin and stained as described in the Materials and methods section. Each set of panels represents identical fields visualized under the FITC (SI) and rhodamine (cathepsin D) filter settings. Bar = 25 μm.

the ER in intestinal cell differentiation because of its key function in the correct folding, post-translational modification and oligomerization of secretory and membrane proteins [19–21,25,27]. The ER is known to exert a general 'quality control' that determines which proteins meet the criteria for transport to the Golgi complex, and is the site of degradation of incorrectly folded proteins [28,29]. This type of protein degradation is energy-dependent, insensitive to inhibitors of lysosomal functions [43], and is also important in the regulation of cholesterol and lipoprotein metabolism [44]. While it is generally recognized that the process of quality control in the ER can prevent secretion or accumulation of abnormal proteins, its role in the regulation of intracellular trafficking, cell polarization and differentiation is still poorly defined.

Hauri and his co-workers have clearly established that brush-border enzymes leave the ER at widely different rates in Caco-2 cells [10,17,18], suggesting that proteins such as SI and lactase, which have longer residence times, may interact more strongly with resident luminal ER proteins, such as chaperones, that are involved in their correct folding [21,24,25]. Oligomerization has

also been found to be essential for exit from the ER in the case of viral glycoproteins [19,23], but this does not appear to be true for brush-border enzymes: SI is present as a monomer both intracellularly and at the cell surface [1,45], and dimerization of DPPIV was shown to occur only during transit through the Golgi [4].

In our studies, heat shock affected selectively and specifically the biosynthesis and cell-surface delivery of SI, and its main effects appeared to be centred on ER-related events. The marked inhibition of the formation of the more mature hmP2 SI precursor could be related to misfolding or instability of the hmP1 form. It has been suggested that conditions of stress, such as high temperature, may perturb the normal folding pathway of a protein, reducing the sites available for interaction of the polypeptide with stress-70 proteins such as BiP and rendering their action less effective [21]. Although not observed in this or previous studies [1,10], putative binding proteins of molecular mass 66 and 82 kDa were found to co-precipitate with SI and DPPIV from cell lysates treated with apyrase (to hydrolyse endogenous ATP) during the first 60–90 min of chase in experiments performed as described in Figure 4 (A. Quaroni, unpublished work), but their nature and function will have to be further evaluated. It has also been suggested that stress-70 proteins may bind different nascent proteins or different polypeptide segments with a wide spectrum of affinities [21], and low-affinity binding may be reversed quickly and spontaneously, thus escaping detection under the conditions we have used in this study for lysis of cells and immunoprecipitation of the brush-border enzymes. The higher temperature could also have affected directly the stability of putative chaperone(s) involved in SI folding and conformational maturation. The cytosolic hsp70, for example, has been shown to undergo a conformational transition with a midpoint at 43 °C and to bind to unfolded proteins in a temperature-dependent manner [46]. Alternatively, the conformational stability of SI itself could have been altered. Heat inactivation experiments employing human intestinal homogenates have shown that the isomaltase activity is inactivated much more rapidly than sucrase activity so that, at 45 °C and neutral pH, 70 % of isomaltase was inactivated in 1 h. The sucrase activity was not affected at all under these conditions [47].

Pulse–chase experiments clearly demonstrated, in cells cultured at 42.5 °C, a longer persistence of SI in an endo-H-sensitive form (Figure 6), increasing its exposure to ER-resident degradative systems [27–29]. This probably contributed to the marked reduction in immunoprecipitable SI at late chase times (Figure 7). The precise site of degradation is, however, uncertain. Recent data have demonstrated that different ER markers do not always co-localize, and this organelle can be subdivided into multiple subcompartments, sometimes with apparently antithetic functions [44]. The lag we have observed between synthesis and degradation of SI (Figure 7) might have reflected delivery to a specialized subcompartment.

In heat-shocked cells, a significant fraction of newly synthesized SI was converted into complex-glycosylated precursor, indicative of passage through the Golgi complex; however, this form of SI cP lacked the epitope defined by the conformation-sensitive monoclonal antibody HSI-5. Thus it was immunologically (and conformationally?) related to the form of SI that we have previously identified in the undifferentiated intestinal crypt cells *in vivo* [1]. Its lack of insertion into the surface membrane suggests that a luminal sorting signal was not present in this improperly folded SI. Evidence has been obtained from the analysis of mutant forms of SI in humans that such a signal is located in the isomaltase subunit [4], and may have been masked or absent in most of the cP produced by heat-shocked cells. The sorting machinery in these cells appeared to function normally, since the polarized cell-surface delivery of DPPIV was not significantly affected (Figures 5 and 7).

Immunofluorescence staining of heat-shocked cells, either intact (Figure 2) or permeabilized with detergents (Figure 8), did not reveal any intracellular accumulation of SI in lysosomes or other organelles. Therefore it can be assumed that the improperly folded cP produced was degraded intracellularly within a few hours of its synthesis, as also indicated by the pulse–chase studies (Figure 7). The most likely site for this degradation is (a portion of) the Golgi complex, as demonstrated for some mutant forms of the enzyme in cases of congenital SI deficiency in humans [6].

There is evidence to suggest that the regulatory steps and processes uncovered by heat shock in Caco-2 cells are also operative in the intestinal mucosa *in vivo*. Incorporation of amino acid analogues into nascent proteins in pig intestinal explants led to a slowdown in the transport of brush-border enzymes to the apical membrane, and caused an accumulation of their high-mannose-glycosylated forms in a pre-Golgi compartment [48]. Similarly, fructose, which induces a defective co-translational high-mannose glycosylation, increased the half-time of the folding of polypeptides, leading to their retention in the ER and eventual intracellular degradation [49]. The rate of SI processing from the ER and Golgi complex to the brush-border membrane was also found to be sensitive to the feeding status of the animal [50]. Intracellular transport and maturation of lactase–phlorizin hydrolase was shown to be retarded in some adults with lactose intolerance, leading to an accumulation of newly synthesized enzyme in the Golgi and a failure to reach the brush-border membrane [51]. These processes may therefore represent a general mechanism for the regulation of the expression of differentiated characteristics by intestinal epithelial cells.

The phenomena reported here raise questions to be addressed in future studies. Among these are the identification of putative chaperones involved in the conformational maturation of brush-border enzymes in the ER, their mechanism of action, the precise sites of intracellular degradation of improperly folded or mutant forms of the enzymes, and the role of the Golgi complex in the final maturation of the enzymes and their delivery to the surface membrane.

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