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Clonal analysis of sucrase–isomaltase expression in the human colon adenocarcinoma Caco-2 cells

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To investigate the biosynthetic basis for the mosaic expression of brush border enzymes in confluent Caco-2 cells, a human colon carcinoma cell line exhibiting characteristics of adult small intestinal enterocytes, we have obtained a series of clones differing markedly in their growth rates, amounts of transforming growth factor-α/epidermal growth factor-like activity released into the culture medium, and sucrase–isomaltase (SI) activity. Other intestinal markers (aminopeptidase N, dipeptidylpeptidase IV, lactase, alkaline phosphatase and 'crypt cell antigen') displayed a much more limited variability in expression, suggesting that the Caco-2 cell clones we have obtained did not differ in their overall ability to differentiate. Immunofluorescence staining, metabolic labelling with radioactive methionine and hybridization analysis of SI mRNA abundance were used to investigate SI synthesis and its regulation in clones endowed with low, intermediate or high sucrase activity. The results obtained have demonstrated heterogeneous SI expression, even in clonal cell lines, and a negative correlation between SI expression and growth factor concentrations in the culture medium, suggesting an autocrine regulation of cell proliferation and differentiation in confluent Caco-2 cells. Pulse–chase experiments using the two clones endowed with the lowest and highest levels of SI activity, followed by immunoprecipitation of labelled SI with epitope-specific antibodies and SDS/PAGE analysis, suggested that both transcriptional and post-translational mechanisms play a role in the regulation of SI expression in intestinal cells.

INTRODUCTION

The human colon tumour cell line Caco-2 represents an excellent *in vitro* model system for the study of intestinal cell differentiation and its regulation. These cells were initially established from a colonic adenocarcinoma by Fogh *et al.* [1] and, under standard culture conditions, form a confluent monolayer consisting of well polarized columnar cells displaying a brush border and tight junctions at their apical aspect [2]. In confluent cultures, differentiation is marked by the expression of disaccharidases and peptidases [sucrase–isomaltase (SI), aminopeptidase N (AP), dipeptidylpeptidase IV (DPP), lactase] [3–5] which are typical components of the mature small intestinal enterocytes, but which are also present in fetal human colon between 11 weeks of gestation and birth [6–8]. During the past few years, these cells have been used advantageously to study various intestinal cell functions, such as the physiology of transporting epithelia and its control [9,10], the synthesis and heparin-stimulated release of diamine oxidase [11], lipoprotein synthesis and secretion [12], and the biosynthesis, processing and intracellular transport of membrane glycoproteins in a polarized epithelium [4,5,13–17].

The mechanisms which trigger and modulate expression of differentiated characteristics in Caco-2 cells upon reaching confluence, and in the intestinal mucosa *in vivo*, are still largely unknown. Recent studies centred on SI and lactase expression during pre- and post-natal development, during crypt-to-villus cell differentiation in adult animals and in Caco-2 and HT-29 cells have provided evidence for both transcriptional and post-translational control sites. In the case of SI, its mRNA and enzyme activity were first detectable, and subsequently increased in parallel during spontaneous or precociously-induced appearance, in newborn rabbits [18] and rats [19] and in human embryos [20], providing evidence for a transcriptional level of regulation. However, Leeper & Henning [19] have suggested that in rats older than 24 days other mechanisms may also be important. In the adult human small intestine [5] and colon [21], crypt cells were found to express a form of the enzyme that is immunologically and conformationally distinct from that present in the brush border membrane of mature enterocytes, suggesting that post-translational processing of SI may play an important role in modulating its expression with cell differentiation. In Caco-2 cells, monensin [22,23] and forskolin [3,24] were found to inhibit SI synthesis primarily at the mRNA level. In the case of lactase, most studies conducted to date [25–28], with one notable exception [29], have demonstrated a marked discrepancy between the post-weaning decline in enzyme activity and the intestinal lactase mRNA levels in the same animals. Similarly, relatively high levels of lactase mRNA were observed in some humans with adult-type hypolactasia [25], again indicating a post-transcriptional control of lactase expression. Alterations in intracellular lactase processing with age or in deficient individuals have also been reported [30–33].

Post-confluent Caco-2 cells display a remarkable heterogeneity in their differentiated characteristics, which is particularly striking in the case of SI: immunofluorescence staining with monoclonal and polyclonal antibodies has consistently revealed the presence of strongly positive and negative neighbouring cells even in cultures kept in a confluent state for several weeks [2,4,13]. In contrast, the same cells appeared to be homogeneously stained with antibodies specific for DPP and crypt cell antigen ([34]; A. Quaroni, unpublished work). These findings are reminiscent of the mosaic pattern of lactase expression demonstrated in a large number of humans with adult-type hypolactasia [35]. To

Abbreviations used: AP, aminopeptidase N; cP, complex-glycosylated sucrase–isomaltase precursor; DMEM, Dulbecco's modified Eagle medium; DPP, dipeptidylpeptidase IV; EGF, epidermal growth factor; FCS, fetal calf serum; hmF, high-mannose form; hmP, sucrase–isomaltase precursor containing glycosidic chains of the hm type; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; SI, sucrase–isomaltase; SSC, standard saline citrate (0.15 M-NaCl, 15 mM-sodium citrate, pH 7.0); TGF, transforming growth factor.

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investigate the factors that regulate SI expression in intestinal cells and are responsible for its mosaic distribution in confluent cultures of Caco-2 cells, we have cloned the original ATCC cell line and characterized the clones obtained with respect to their relative SI mRNA levels, biosynthesis and intracellular processing of SI, and amounts of transforming growth factor-α (TGF)/epidermal growth factor (EGF)-like activity secreted into the culture medium. The results obtained indicate that SI expression may be regulated at both the transcriptional and post-translational levels, and growth factors synthesized and secreted into the culture medium may play an important role in the differentiation of Caco-2 cells.

MATERIAL AND METHODS

Materials

Balb/c mice (15–17 g) were obtained from Charles River Breeding Laboratory (Wilmington, MA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), irradiated fetal calf serum (FCS), penicillin/streptomycin mixture and trypsin (2.5% in Hanks balanced salt solution without calcium and magnesium) were obtained from Whittaker Biosciences (Walkersville, MD, U.S.A.). Tris, Hepes, phenylmethanesulphonyl fluoride (PMSF), aprotinin, leupeptin and antipain were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); acrylamide and bisacrylamide were from Bio-Rad Laboratories (Richmond, CA, U.S.A.); Sequenal-grade SDS was from Pierce Chemical Co. (Rockford, IL, U.S.A.); Protein A–Sepharose CL 4B and CNBr-activated Sepharose 4B were from Pharmacia LKB Biotechnology (Piscataway, NJ, U.S.A.). Triton X-100, 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 Du Pont/NEN (Boston, MA, U.S.A.). Small volume filtration devices were from Porex Medical (Fairburn, GA, U.S.A.).

Cell culture

The human colonic cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and grown in 100 mm plastic Petri dishes (Falcon; Becton–Dickinson Labware, Oxnard, CA, U.S.A.), at 37 °C in an atmosphere of 95% air and 5% CO_2 in DMEM containing 10% (v/v) FCS, 50 units of penicillin/ml, 50 μg of streptomycin/ml, 20 mM-Hepes and 4 mM-glutamine. The cultures were replaced twice weekly with 10 ml of fresh medium and subcultured serially when approx. 80% confluent. Double-cloned cell lines were obtained by dilution plating and were named Caco-2/1–16. They were routinely screened for the absence of mycoplasma contamination and tested for phenotype stability (determination of sucrase activity) between passages 5 and 20, which were used for the experiments described in this report.

Pulse–chase and metabolic labelling

Labelling of Caco-2 cells was performed on late-confluent cells (15 days post-confluence). Dishes were rinsed with 2 × 10 ml of methionine-free medium [methionine-free DMEM (Gibco Laboratories Inc., Grand Island, NY, U.S.A.) containing 5% dialysed FCS and antibiotics and glutamine as above] and incubated for 1 h with 10 ml of the same medium. Then the medium was replaced with 3 ml per dish of methionine-free medium containing 250–300 μCi of [^{35}S]methionine/ml (1083–1151 Ci/mmol) and the incubation was continued for 1 h (pulse–chase experiments) or 6 h (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 10 mM unlabelled methionine for up to 24 h. To harvest the cells, dishes were then rinsed with 10 ml of phosphate-buffered saline (PBS; (10 mM-sodium phosphate/150 mM-NaCl, pH 7.4)) and cells were scraped with a rubber policeman into homogenization buffer (2 mM-Tris/HCl, pH 7.4, and 50 mM-mannitol) supplemented with protease inhibitors (1 mM-PMSF, 50 μg of leupeptin/ml, 50 μg of antipain/ml, 0.1 mg of aprotinin/ml) and homogenized in a glass/Teflon Potter–Elvehjem homogenizer (25 strokes with a motor-driven pestle). Following centrifugation at 950 *g* under refrigeration, the supernatant (post-nuclear lysate) was supplemented with 200 mM-NaCl and 1% Triton X-100, and incubated with monoclonal antibodies bound to Sepharose 4B beads as previously described [34].

Membrane purification

Apical (brush border) membranes were purified from Caco-2 cell homogenates by the method of Kessler *et al.* [36]. A crude total membrane fraction was obtained as follows. Cells were washed 3 times with PBS and homogenized in a glass/Teflon Potter–Elvehjem homogenizer in 2 mM-Tris/50 mM-mannitol. Homogenates were centrifuged at 2700 *g* for 10 min and supernatants were spun at 207000 $g_{av.}$ for 30 min. Resulting pellets were designated 'crude membrane fractions'. A mixture of protease inhibitors (see above) was added to all buffers and solutions used for homogenization and membrane purification. Proteins were determined by the method of Lowry *et al.* [37] and mebrane preparations were monitored for purification [38] by measuring the increase in the specific activity of sucrase, which was 15–20 times higher in the final microvillus membrane fraction than in the homogenate.

Enzyme assays

Enzyme assays were carried out as follows: sucrase, maltase and lactase by the method of Messier & Dahlqvist [39], with the appropriate substrates (0.1 M in all cases); AP according to Roncari & Zuber [40] with L-leucine-*p*-nitroanilide as substrate; DPP with glycyl-L-proline-*p*-nitroanalide-*p*-tosylate as substrate, and alkaline phosphatase by the method of Forstner *et al.* [41] with *p*-nitrophenylphosphate as substrate.

Growth curves and determination of TGF-α/EGF-like activity in serum-free conditioned media

Cells were seeded in 60 mm dishes at 8×10^4 cells/dish and cultured in complete medium or in medium containing 10% FCS; cells in triplicate dishes were used for determination of cellular DNA at daily intervals. The number of cells/dish was calculated using the conversion factor of 19.33 μg of DNA/10^6 cells, determined using subconfluent cultures of Caco-2/15 cells. This procedure was made necessary because of the difficulty encounted with preparation and counting of single cell suspensions from confluent cultures which, after trypsin treatment, produced large clumps of cells. DNA was assayed in total cell homogenates using a method based on the enhancement of fluorescence seen when bisbenzimidazole (Hoechst 33258) binds to DNA [42]. Cells were homogenized with a Polytron (Brinkmann Instruments, Westbury, NY, U.S.A.) in phosphate/saline buffer (50 mM-sodium phosphate/2 M-NaCl, pH 7.4). An aliquot of the homogenate (usually 10–50 μl) was added to 2 ml of 10 mM-Tris/HCl buffer/100 mM-NaCl, pH 7.4, containing 0.1 μg of Hoechst 33258 dye/ml. Calf thymus DNA was used as a standard. Fluorescence was measured with a dedicated minifluorimeter (TKO 100; Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) according to the protocol suggested by the manufacturer.

Serum-free conditioned media from each Caco-2 cell clone were collected from groups of 6×100 mm dishes [a total of $(1.2–1.5) \times 10^8$ cells] over a 24 h period. Cells were preincubated overnight with serum-free medium to remove any trace of serum components before starting collection. The radioreceptor assay for TGF-α/EGF like activity was performed as described by Anzano *et al.* [43]

Gel electrophoresis and immunoblotting

SDS/10% PAGE was performed according to Thomas & Kornberg [44]. Labelled proteins were detected by fluorography as previously described [45].

Immunoblotting experiments were performed essentially according to the procedure already described [5]. Briefly, proteins from SDS/polyacrylamide gels were electrophoretically transferred to a nitrocellulose membrane, which was subsequently blocked in PBS containing 5% Blotto and incubated with monoclonal antibodies (ascitic fluid, diluted 1:500). Membranes were then washed, incubated with alkaline phosphatase-conjugated goat anti-[mouse IgG (H+L)] antibody (Promega Biotec, Madison, WI, U.S.A.), further washed and finally incubated with a chromogenic substrate for alkaline phosphatase detection (Promega Biotec).

Monoclonal antibodies

Production and characterization of monoclonal antibodies specific for crypt cell antigen (FBB2/29, [46]) and human SI (designated HSI antibodies), namely HSI-5, HSI-9 and HSI-14 [5], used in the present work has been described elsewhere; FBB2/29 is an IgM, HSI-5 and HSI-14 are of the IgG_1 subtype, and HSI-9 is of subtype IgG_{2b}.

The monoclonal antibody to AP (HBB2/45) used in this study was produced from a mouse immunized with brush border membrane purified from human jejunum by the hybridoma technique, as described previously for antibodies to rat intestinal membrane antigens [45]. Its antigen specificity was determined as described for antibodies to human SI [5]. Briefly, HBB2/45 was purified from hybridoma conditioned medium and ascites fluid by affinity chromatography on a Protein A–Sepharose CL 4B column and covalently bound to CNBr-activated Sepharose 4B beads [34]. The antibody–beads conjugate was: (1) tested for its ability to bind various marker enzymes (sucrase, maltase, lactase, AP, DPP, alkaline phosphatase) present in Triton X-100-solubilized brush border membrane obtained from human jejunum; only AP activity was detected; and (2) incubated with Triton X-100-solubilized ^{14}C-labelled brush border membrane proteins [5]. The bound, labelled antigen was analysed by SDS/PAGE, and two bands of apparent molecular mass 150 and 300 kDa were observed, corresponding respectively to the monomeric and aggregated (dimeric) forms of AP [4].

Immunofluorescence staining

Post-confluent Caco-2 cells (12 days) grown in 35 mm dishes were rinsed with PBS, fixed with 2% formaldehyde and stained by the double-antibody fluorescence technique as described [47]. Non-immune mouse serum was used as a negative control.

Radioimmunobinding assay

Binding of monoclonal antibodies to different clones of the Caco-2 cell line was performed as described previously [48] with the use of ^{125}I-labelled sheep anti-mouse IgG [$(F(ab')_2$ fragment] as second antibody. Approx. 1×10^6 c.p.m. of ^{125}I was added to each dish; parallel cultures were used for protein determination. Cells were tested between 12 and 15 days of confluence.

Preparation and analysis of RNA

Total RNA was isolated from Caco-2 cell clones using the guanidinium thiocyanate/caesium chloride procedure [49]. Poly(A)$^+$ RNA was purified by using a Poly(A) Quik mRNA purification kit from Stratagene (La Jolla, CA, U.S.A.), following the instructions supplied by the manufacturer. For hybridization analyses, RNA was denatured with formaldehyde (6.6%) and resolved by electrophoresis on 1.2% agarose/formaldehyde gels [50]. Electroblotting of RNA–agarose gels on to Gene-Screen Plus membranes was performed as recommended by the supplier (Du Pont–NEN). Following transfer, the RNA was irreversibly cross-linked to the membrane by u.v. irradiation (Stratalinker, Stratagene) as described [51]. Prehybridizations (3 h) and hybridizations (18 h) were performed at 42 °C in 50% formamide/1% SDS/1 M-NaCl/10% dextran sulphate and contained 100 mg of denatured salmon sperm DNA/ml. Labelled probes were then denatured by boiling and were added directly to the prehybridization solution to achieve final concentrations of approx. 5–10 ng of probe DNA/ml ($10^7–10^8$ d.p.m./μg). Following hybridization, the membranes were washed at various stringencies ($0.1 \times$ SSC when using the SI probe, $1 \times$ SSC with the actin probe, always in the presence of 1% SDS) at 60 °C for 1 h, blotted dry and exposed to Kodak XAR-5 film with an intensifying screen for 4–12 h at −70 °C. The intensity of the bands was assessed by using a laser densitometer (Ultroscan XL; Pharmacia LKB).

cDNA probes

The plasmid pSI2 [52] containing a 2000 bp insert comprising about one-third of the coding region of human SI cDNA and 5 nucleotides of 5′-untranslated sequence was obtained from Dr. D. Swallow (MRC Human Biochemical Genetics Unit, University College, London, U.K.).

A *Hin*dIII restriction fragment (1700 bp) from exon 2 of the *Drosophila* actin 5C gene (E. Keller, Cornell University) was amplified in pUC18 and subsequently used as an actin probe for RNA hybridization analyses.

The cDNA probes were isolated from vector DNA by appropriate restriction endonuclease digestion and agarose gel resolution [53]. The actin probe was labelled by nick-translation [54], and the SI probe was labelled using the Random Primers DNA Labelling System from BRL-Life technologies (Gaithersburg, MD, U.S.A.), both in the presence of [α-^{32}P]dCTP.

Statistical analysis

Student's *t* test for unpaired data was used as a statistical test. Results, expressed as means ± S.D., were considered significant when $P < 0.05$.

RESULTS

Characterization of Caco-2 cell clones

Caco-2 cells were obtained from the American Type Culture Collection at passage 17 and were cloned by dilution plating. Individual wells of the cloning plates were visually checked after 1 week from plating to ensure the presence of single clones. Following serial expansion, 16 clones were tested in a post-confluent state (12 days) for sucrase activity and binding of monoclonal antibodies specific for SI (HSI-14), AP (HBB2/45), and crypt cell antigen (FBBS/29), a previously described marker for small intestinal crypt cells and colonic tumour cells both *in vivo* and *in vitro* [34,46,48,55]. The results, summarized in Table 1, demonstrated a great variability in the amount of sucrase activity present in brush border membranes purified from the

Table 1. Caco-2 cell clones: sucrase activity and binding of monoclonal antibodies

Sucrase activity was determined on brush border membranes purified from cells confluent for 12 days, and is expressed as munits of enzyme activity per mg of protein. Binding of monoclonal antibodies to cells cultured in 60 mm diameter dishes and confluent for 15 days was measured as described in the Materials and methods section; the antibodies used were specific for SI (HSI-9), AP (HBB2/45) and crypt cell antigen (FBB2/29). Values are means ± S.E.M., $n = 3$.

Clone no.	Sucrase activity (munits/mg of protein)	^{125}I-Antibody bound (c.p.m./mg of protein)		
		HSI-9	HBB2/45	FBB2/29
1	27.5±4.1	2156±13	8281±153	49406±1839
2	32.3±9.4	3140±87	6815±89	47237±2641
3	24.6±6.2	2004±56	7316±273	35381±3312
4	46.4±7.1	8837±81	9653±67	69739±210
5	110.2±17.6	19234±174	7546±71	92541±164
6	267.7±24.2	34449±1435	7983±45	64534±1056
7	27.8±2.1	4885±388	5976±174	42303±4877
8	26.6±3.9	4490±212	6932±117	51629±368
9	36.9±6.3	6961±584	7985±654	69468±2106
10	182.4±11.6	18735±2652	8812±178	41982±861
11	31.8±1.3	7684±194	8001±48	55479±1202
12	138.5±6.4	17441±763	5985±112	53223±732
13	221.3±17.9	29942±1179	8655±321	41967±2687
14	136.8±9.8	18932±741	8443±70	50337±337
15	273.0±16.9	46887±1027	8657±234	42332±2266
16	101.4±5.6	13554±427	7580±137	38705±2300

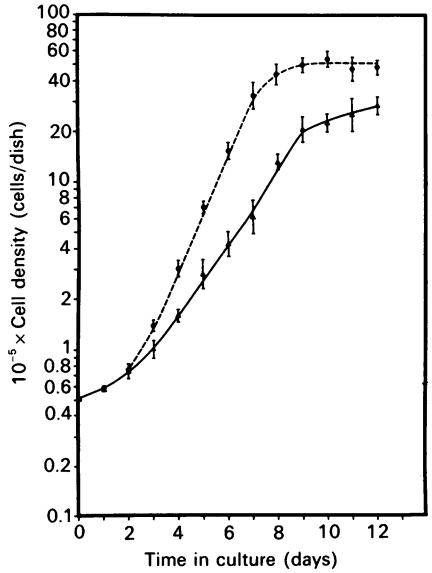

Fig. 1. Growth curves of Caco-2/3 and Caco-2/15 cells cultured in standard complete medium

Cells were seeded in 60 mm dishes at 5×10^4 cells/dish on day 0, and triplicate dishes were used for cellular DNA determination at daily intervals. Cell numbers/dish were calculated using a conversion factor of 19.33 μg of DNA/10^6 cells. Confluent monolayers were observed at 8–9 days after seeding. ●, Caco-2/3 cells; ▲, Caco-2/15 cells. Results represent means ± S.E.M.

different clones (ranging between 24.6 and 273 munits/mg of protein for clones 3 and 15 respectively, an 11-fold difference). This was also reflected in the amounts of anti-SI antibody which bound to the cell monolayers (varying between 2000 and 46900 c.p.m./mg of cell protein for clones 3 and 15 respectively, a 23-fold difference). In contrast, the antibody to AP showed equivalent binding to all clones tested. The amount of crypt cell antigen expressed showed only a 2.6-fold variation among different clones, and did not correlate with SI activity levels (for example, clones 3 and 15, the lowest and highest SI producers, bound very similar amounts of FBB2/29 antibody).

Sucrase expression by the different Caco-2 cell clones was, in general, negatively correlated with their growth rate. As an example, the growth curves for clones Caco-2/3 and Caco-2/15 are compared in Fig. 1: their population doubling times during the logarithmic phase of growth were estimated as 24 and 36 h respectively. The apparent saturation density of these (and other) clones was also negatively correlated with the amounts of SI produced, being greater in faster-growing clones (Fig. 1). A possible explanation for these findings is an autocrine stimulation of cell growth with a corresponding inhibition of SI expression, as colon tumour cell lines have been previously shown to synthesize and secrete significant amounts of growth factors into the culture medium [43]. We have therefore selected a group of clones representative of low (clones 1 and 3), intermediate (clone 5) and high (clones 6, 13 and 15) SI producers and determined the amounts of TGF-α/EGF-like activity present in their conditioned media. The results obtained (Table 2) demonstrated an approx. 100-fold difference in growth factor production among different clones, and a negative corelation between growth factor activity and SI expression. Clones Caco-2/1 and Caco-2/3 were by far the highest producers of TGF-α/EGF-like activity, while Caco-2/15 was the lowest.

Immunolocalization of SI and AP in Caco-2 cell clones

Immunofluorescent staining of several Caco-2 cell clones, examined as confluent monolayers or sectioned perpendicularly to their growth surface, demonstrated a marked heterogeneity with respect to both SI and AP expression, similar to that observed in the original cell line before cloning. No reproducible difference was noted among different cultures maintained in a confluent state for comparable periods of time in the percentage of cells stained by the anti-SI antibody. The intensity of the fluorescence was, however, much greater in clones expressing higher levels of sucrase activity (e.g. Figs. 2*e* and 2*f*, comparing clones Caco-2/3 and Caco-2/15). The antibody HBB2/45 against

Table 2. TGF-α/EGF-like activity production by different Caco-2 cell clones

Cells were preincubated overnight with serum-free medium, then serum-free conditioned media were collected over a 24 h period from each Caco-2 cell clone. The radioreceptor assay for TGF-α was performed as described by Anzano *et al.* [43]. Results were normalized to the number of cells present at the end of the collection period. Values are means ± S.E.M., $n = 3$.

Clone no.	TGF-α/EGF activity (ng/10^8 cells)
1	52.1 ± 13.5
3	74.4 ± 8.9
5	9.8 ± 1.6
6	1.4 ± 0.4
13	4.7 ± 1.3
15	0.8 ± 0.2

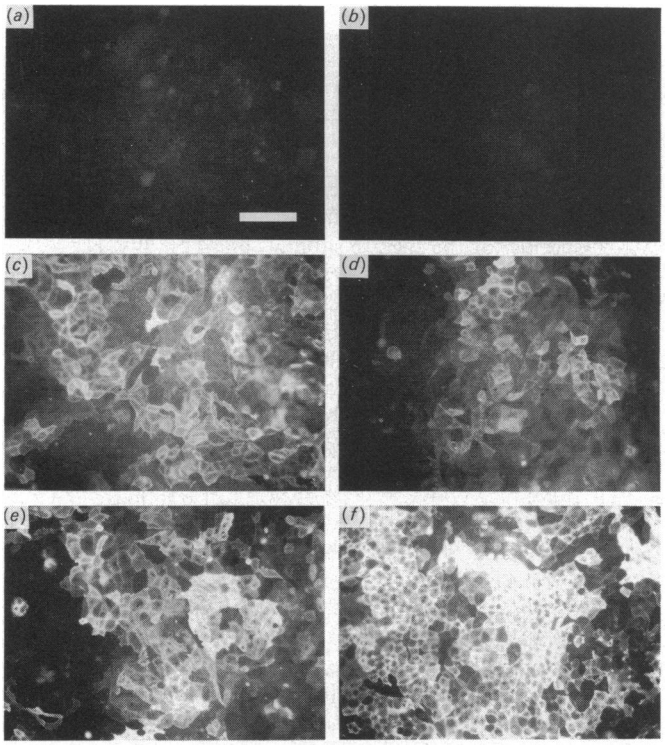

Fig. 2. Indirect immunofluorescence staining of Caco-2/3 (*a,c,e*) and Caco-2/15 (*b,d,f*) cells with monoclonal antibodies specific for AP (*c,d*) and SI (*e,f*)

Cells incubated with non-immune mouse serum (negative controls) are shown in (*a*) and (*b*). Cells were fixed with 2% formaldehyde 12 days after confluence. Bar, 100 μm.

AP produced weaker staining (Figs. 2*c* and 2*d*) of comparable intensity in all clones examined.

Influence of time spent in a confluent state on SI expression

For these experiments, we selected clones Caco-2/3 and Caco-2/15, expressing the lowest and highest levels of sucrase activity respectively. The two clones were grown in parallel under identical culture conditions and were harvested at various times (0–25 days) after they formed confluent monolayers. Brush border membranes were purified and used to evaluate SI expression by determination of sucrase activity and detection of immunoreactive enzyme on immunoblots.

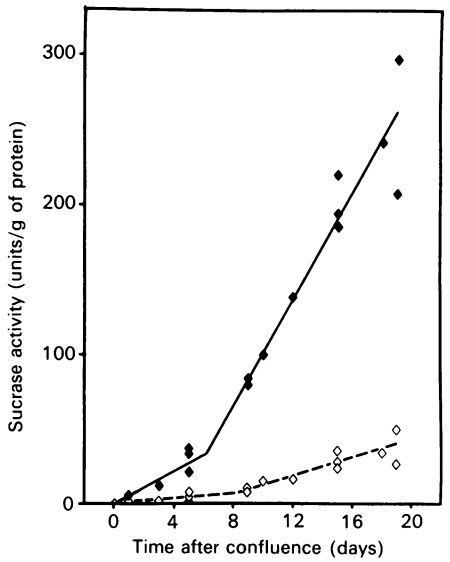

Fig. 3. Increases in sucrase activity in post-confluent Caco-2/3 (◇) and Caco-2/15 (◆) cultures

Sucrase activity was determined on brush-border-enriched fractions and normalized to membrane protein determined by the method of Lowry *et al.* [37]. The data presented are the individual values obtained in four separate experiments.

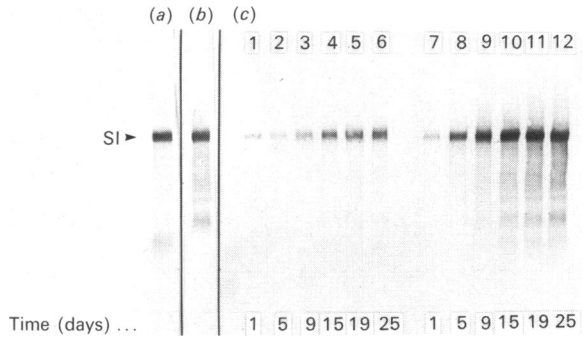

Fig. 4. Immunoreactive SI in post-confluent Caco-2/3 and Caco-2/15 cultures

Brush border membranes were prepared from cells harvested between 1 and 25 days after reaching confluence. For each sample, 50 μg of total brush border proteins were separated by SDS/PAGE and electrophoretically transferred to a nitrocellulose membrane. SI-related polypeptides were detected by incubation with monoclonal antibodies HSI-9 (sucrase-subunit-specific) and HSI-14 (isomaltase-subunit-specific), followed by anti-mouse IgG conjugated to alkaline phosphatase (second antibody) and an alkaline phosphatase chromogenic substrate. (*a*, *b*) Same sample of brush border membrane proteins incubated with, respectively, antibodies HSI-9 and HSI-14 alone. (*c*) Samples, incubated with an equal mixture of HSI-9 and HSI-14, obtained from Caco-2/3 (lanes 1–6) and Caco 2/15 (lanes 7–12) cells. Days after confluence are indicated. SI refers to the position of cP SI precursor.

Sucrase activity (Fig. 3) was very low in both Caco-2/3 and Caco-2/15 cells at the time they reached confluence (day 0). After 6–8 days it increased linearly with time, but at very different rates in the two clones. At 19 days post-confluence, sucrase specific activity (munits/mg of protein) in purified brush border membranes was approx. 6.6 times greater in Caco-2/15 cells (Caco-2/3, 34.2 ± 3.9; Caco-2/15; 225.7 ± 17.5, means ± S.E.M., $P < 0.005$).

To determine whether the differences in sucrase activity

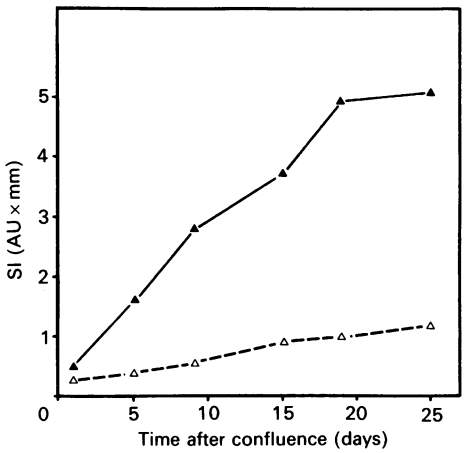

Fig. 5. Densitometric analysis of the immunoblot shown in Fig. 4(*c*)

The output (peak areas) of the laser densitometer used is expressed in absorbance unit (AU) times mm. △, Caco-2/3; ▲, Caco-2/15.

Table 3. Activities of lactase, AP, DPP and alkaline phosphatase in brush-border-enriched fractions isolated from late-confluent (15–19 days) Caco-2/3 and Caco-2/15 cells

Data represent means ± S.E.M. of five separate determinations (three at 15 days and two at 19 days post-confluence). Differences in lactase, DPP and alkaline phosphatase activities between clone 3 and clone 15 were significant by paired *t* test, * $P < 0.005$.

Clone no.	Enzyme activity (units/g of protein)			
	Lactase	AP	DPP	Alkaline phosphatase
Caco-2/3	6.97 ± 0.61	23.48 ± 0.94	692.8 ± 48.2	343.4 ± 30.1
Caco-2/15	10.98 ± 0.66*	21.18 ± 0.77	1026.5 ± 66.3*	789.2 ± 54.2*

Table 4. SI synthesis by different clones of Caco-2 cells

Caco-2 cells confluent for 15 days were labelled with [^{35}S]methionine for 1 h. Labelled SI was immunoprecipitated from Triton X-100-solubilized total cell membranes with a mixture of antibodies HSI-9 and HSI-14, analysed by SDS/PAGE under reducing conditions (50 mM-dithiothreitol), and visualized by fluorography. The fluorographs were scanned with a laser densitometer, and the output (peak areas) is expressed as the product of absorbance units (AU) and millimeters (mm). Values represent means ± S.E.M. of three cultures.

Clone no.	SI (AU × mm)
1	5.31 ± 0.18
3	4.78 ± 0.24
5	9.96 ± 0.50
6	10.54 ± 0.91
13	10.84 ± 0.63
15	11.83 ± 0.38

observed between the two clones could be attributed to expression of partially inactive forms of the enzyme [5,21], we quantified immunoreactive SI by immunoblotting in membranes purified from Caco-2/3 and Caco-2/15 cells at 1–25 days post-confluence (Fig. 4). Brush border membrane proteins (50 μg/sample) were

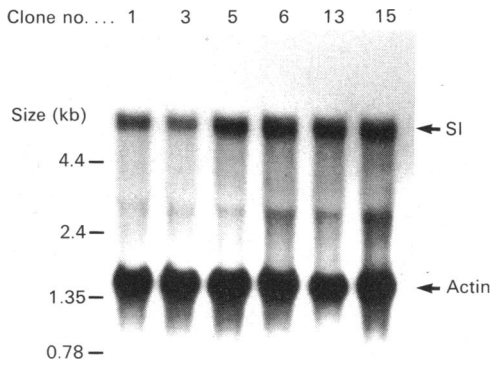

Fig. 6. RNA transfer hybridization of poly(A)$^+$ RNA extracted from 15-days-confluent Caco-2 cell clones

Clone numbers are indicated above the lanes. RNA (0.5 μg/lane) was denatured and resolved on a 1.2% agarose/formaldehyde gel, then electrophoretically transferred to a GeneScreen Plus membrane, u.v.-cross-linked and hybridized to the ^{32}P-labelled SI and actin cDNA probes. Following hybridization and washing (see the Materials and methods section for details) the membrane was exposed to a Kodak XAR-5 film with an intensifying screen for 2 days at −70 °C.

separated by SDS/PAGE, electrophoretically transferred to nitrocellulose and incubated with an equal mixture of antibodies HSI-9 (specific for the sucrase subunit) and HSI-14 (specific for the isomaltase subunit) [5]. From the immunoblots obtained (Fig. 4) and the corresponding densitometric analysis (Fig. 5), it is apparent that the relative amounts of immunoreactive SI, barely detectable 1 day after confluence, increased almost linearly thereafter and displayed quantitative differences between the two clones comparable with those observed in the sucrase assay. The amount of SI accumulated in brush border membranes from Caco-2/15 cells at 19–25 days post-confluence was 5–6-fold greater than in corresponding Caco-2/3 cell fractions.

Expression of other microvillar hydrolases

The results obtained in the immunobinding assay using antibodies to AP and crypt cell antigen (Table 1) suggested that the marked differences observed in SI activity reflect regulation of expression of this enzyme rather than alterations in the overall ability of the various Caco-2 cell clones to differentiate. This was further substantiated by analysis of the entire pattern of enzyme activities in brush border membranes purified from confluent (15–19 days) Caco-2/3 and Caco-2/15 cells. As shown in Table 3, the activities of lactase, DPP and alkaline phosphatase were significantly higher in Caco-2/15 cells ($P < 0.005$), but the specific activity ratios between membranes from Caco-2/15 and Caco-2/3 cells were only 1.6 for lactase, 1.7 for DPP and 2.3 for alkaline phosphatase, much lower than the corresponding values (6–11) for sucrase observed in the same cells. AP activity was actually slightly higher in membranes from Caco-2/3 cells, although the difference did not reach statistical significance.

Biosynthesis of SI and AP

To identify the regulatory sites and biosynthetic step(s) responsible for the marked difference in SI expression among the Caco-2 cell clones, we first evaluated their ability to synthesize this enzyme over a 1 h period, during which SI has been previously shown to be present exclusively as a high-mannose precursor (hmP) in the endoplasmic reticulum [4,5,13]. The same group of clones previously tested for growth factor production (Table 2) was used in this study. Following the 1 h pulse with

[^{35}S]methionine, labelled SI was immunoprecipitated with a mixture of antibodies HSI-9 and HSI-14 from total cell membranes solubilized with Triton X-100. The results obtained (Table 4) demonstrated an approx. 2-fold difference in SI biosynthetic activity between clones endowed with the lowest (clones 1 and 3) and highest (clones 6, 13 and 15) sucrase activities. Clone Caco-2/5 synthesized approximately the same amount of labelled SI as clones 6, 13 and 15, in contrast with its 2.5-fold lower sucrase activity as determined on purified brush border membranes (Table 1).

Table 5. SI mRNA levels in different clones of Caco-2 cells

Poly(A)$^+$ RNA (0.5 μg/lane), purified from 15-days-confluent Caco-2 cell clones, was denatured and resolved on a 1.2% agarose/formaldehyde gel, then electrophoretically transferred to a GeneScreen Plus membrane, u.v.-cross-linked, and hybridized to the SI cDNA probe labelled by the random primer extension procedure. Following hybridization, the membrane was washed in 0.1 × SSC containing 1% SDS at 60 °C (3 × 30 min) and exposed to a Kodak XAR-5 film with an intensifying screen for 2 days at −70 °C. Subsequently the membrane was stripped of the SI probe, rehybridized to the actin probe labelled by nick translation, washed in 1 × SSC containing 1% SDS at 60 °C (3 × 30 min), and again exposed to a Kodak XAR-5 film for 12 h at −70 °C. The autoradiographs were scanned with a laser densitometer and the output (peak areas) is expressed as the product of absorbance units (AU) and millimetres (mm). Values represent means ± S.E.M. of three cultures.

Clone no.	SI mRNA (AU × mm)	Actin mRNA (AU × mm)	$\frac{\text{SI mRNA}}{\text{actin mRNA}} \times 100$
1	3.73 ± 0.64	15.3 ± 0.9	24.37
3	3.20 ± 0.30	17.4 ± 0.4	18.39
5	8.92 ± 0.18	20.1 ± 2.3	44.37
6	8.94 ± 1.07	17.1 ± 0.8	52.28
13	9.56 ± 0.75	15.5 ± 1.4	61.67
15	10.70 ± 0.69	20.9 ± 1.1	51.23

The relative abundance of SI mRNA present in the same group of clones was estimated by Northern blot analysis of poly(A$^+$) RNA purified from confluent cultures, and compared with actin mRNA levels used as a reference (see Fig. 6, and Table 5 for a densitometric analysis of results obtained in a different experiment using triplicate samples). The results obtained demonstrated an excellent correlation between SI mRA levels and the relative amounts of SI synthesized over a 1 h period (Table 4): Caco-2/1 and Caco-2/3 cells had approximately half of the SI mRNA abundance of the other clones, which all had similar levels.

Finally, pulse–chase experiments were performed to determine whether clones endowed with low (Caco-2/3) and high (Caco-2/15) sucrase activities differed with respect to processing and/or intracellular degradation of the previously characterized SI biosynthetic forms: the initial co-translationally glycosylated high-mannose precursor hmP_1, the conformationally distinct derivative hmP_2 and the complex glycosylated precursor (cP) formed in the Golgi complex [4,5,13–15]. As in our previous study [5], these forms were distinguished by sequential immunoprecipitation with antibodies HSI-5 (recognizing hmP_2 and cP) and HSI-14 (precipitating the remaining hmP_1), followed by SDS/PAGE analysis. A protein of molecular mass 195 kDa (labelled X in Fig. 7), whose relationship with SI is unknown [5], was also reproducibly precipitated by HSI-14 during the first 3–4 h of chase. Finally, the cell extracts were incubated with the antibody HBB 2/45 to precipitate the complex glycosylated and high-mannose forms of AP, which was used as an internal control for overall protein synthesis (Figs. 7*c* and 7*d*). Cellular extracts containing equivalent amounts of total labelled proteins (25×10^6 c.p.m./extract) were used for both clones. The results obtained are shown in Fig. 7, and the corresponding densitometric analysis in Fig. 8.

Confirming the results obtained by continuous 1 h labelling of the same cells (Table 4), the combined amounts of hmP_1 and hmP_2 immunoprecipitated from Caco-2/15 cells at the end of the pulse period (chase = 0) was approximately twice that synthesized by Caco-2/3 cells. Thereafter the conversion rates among different SI biosynthetic forms were similar or identical in the two clones. After 90–120 min, hmP_1 was nearly completely

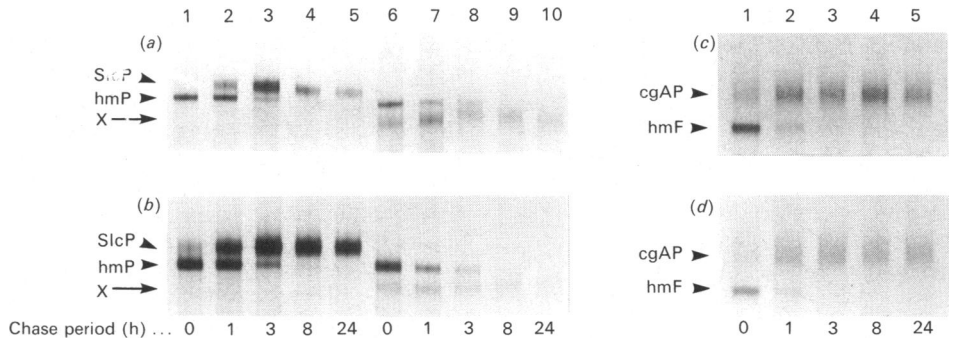

Fig. 7. Pulse-chase labelling of different biosynthetic forms of SI and AP in 15-days-confluent Caco-2/3 (*a*, *c*) and Caco-2/15 (*b*, *d*) cultures

The cells were labelled with [^{35}S]methionine for 1 h, rinsed with complete medium and then incubated with complete medium containing 10 mM unlabelled methionine. At the indicated time (starting from the end of the pulse), cell homogenates supplemented with 1% Triton X-100 and protease inhibitors were sequentially incubated with HSI-5/Sepharose 4B beads (hmP_2 and cP forms of SI precipitated are presented in *a* and *b*, lanes 1–5), followed by two more fresh samples of HSI-5 (which completely depleted the extracts of any HSI-5-reactive SI [5]), and finally with HSI-14 beads (which precipitated the hmP_1 form of SI, *a* and *b*, lanes 6–10). The supernatants were subsequently incubated with HBB2/45-Sepharose 4B beads, which precipitated all forms of AP (*c* and *d*, lanes 1–5). Bound antigens were solubilized from the washed beads with sample solution, separated by SDS/PAGE and visualized by fluorography. At the left margin of (*a*) and (*b*), arrows point to the complex glycosylated form of the SI precursor (SIcP), the high-mannose forms of the precursor (hmP), and to the protein designated X, reproducibly immunoprecipitated by HSI-14 [15]. At the left margin of (*c*) and (*d*), arrows point to the complex glycosylated (cgAP) high-mannose (hmF) forms of AP.

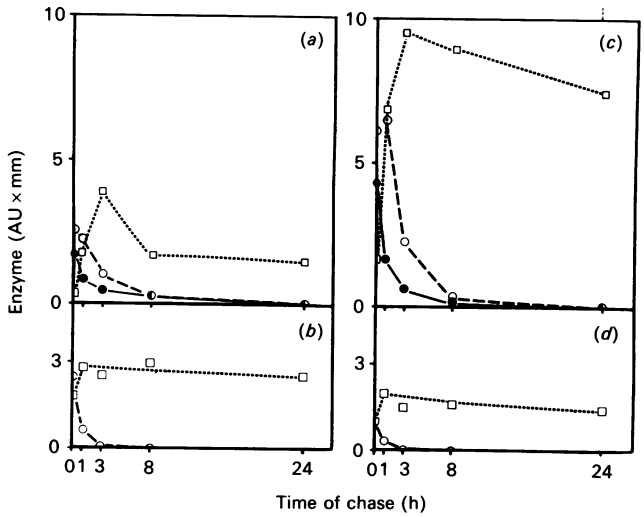

Fig. 8. Densitometric analysis of the fluorograms shown in Fig. 7

(*a*), (*b*) Caco-2/3 cells; (*c*), (*d*) Caco-2/15 cells. The output (peak areas) of the laser densitometer used is expressed in absorbance unit (AU) times mm. (*a*), (*c*) SI: ●, hmP_1; ○, hmP_2; □, cP. (*b*), (*d*) AP: ○, high-mannose form; □, complex glycosylated form.

converted into hmP_2 plus cP, and by 3–4 h of chase cP was the only form of SI detectable. In the process, however, the ratio of the combined amounts of the different SI forms (hmP_1 + hmP_2 + cP) present in Caco-2/15 and Caco-2/3 cells increased to 3.0–3.5 between 1 and 3 h, and to 5.3 and 5.0 at 8 and 24 h of chase respectively. These results indicate that the differences in SI levels observed between these two Caco-2 cell clones could be attributed both to decreased synthesis (2-fold) and to a lesser stability of the hmP and cP forms in Caco-2/3 cells.

In contrast, AP synthesis was about 50% higher in Caco-2/3 cells (Fig. 8) and, once formed, the complex-glycosylated enzyme appeared to be quite stable up to 24 h of chase.

DISCUSSION

The Caco-2 cell clones that we have obtained and characterized exhibited similar morphology and growth characteristics, and differed primarily in their sucrase activity, amount of SI expressed at the apical surface membrane (Table 1) and growth factor concentrations present in their conditioned media (Table 2). Other markers of intestinal cell differentiation were expressed at similar levels in the clones examined (Tables 1 and 3), suggesting that they retained the ability to differentiate upon reaching confluence typical of the parental cell line [2]. Immunofluorescence staining with antibodies to SI and AP (Fig. 2) demonstrated a mosaic pattern of expression unrelated to the enzyme activity levels of individual clones, indicating that heterogeneous expression of differentiated markers is an intrinsic property of Caco-2 cells which may be modulated by local factors or cell–cell interactions.

The marked differences observed among various clones in sucrase activity (spanning an 11-fold range) and immunoreactive cell surface SI, but not in AP and DPP activities, suggest that expression of these brush border enzymes in Caco-2 cells is regulated in different ways. This has also been observed on treatment of these cells with monensin, which resulted in a marked decrease in surcrase synthesis without significantly affecting DPP levels [22]. It is noteworthy that AP and DPP are expressed by the entire intestinal epithelium in rats [56] and humans (A. Quaroni, unpublished work), and are the only two brush border enzymes detected, although at very low levels, on the surface membrane of cultured intestinal crypt cells [57]. They may therefore represent constitutive differentiated markers of intestinal epithelial cells both *in vivo* and in culture.

The negative correlation observed between sucrase activity (Table 1) or synthesis (Table 4) and growth-related characteristics [population doubling times, apparent saturation densities (Fig. 1)] of the different clones indicates that growth factors such as EGF and TGF-β [43] may be important autocrine regulators of SI expression in Caco-2 cells. These and other colon tumour cell lines have been found to synthesize and secrete into the culture medium significant amounts of EGF/TGF-α, TGF-β and platelet-derived growth factor [43,58], and to express the corresponding receptors on their surface membrane [59]. HT-29 cells, which synthesize SI but rapidly degrade it intracellularly when grown in a glucose-containing medium [60], produce relatively large amounts of TGF-α and TGF-β [43]. These cells can be induced to express SI on their cell surface by suramin [61], a drug which inhibits binding of several growth factors to their cellular receptors [62]. The availability of Caco-2 clones producing limited amounts of endogenous growth factors should allow a direct test of this hypothesis.

The structure and biosynthesis of SI have been investigated in great detail [63], making it an excellent marker enzyme for the study of the mechanisms regulating intestinal cell differentiation. In the brush border membrane of adult enterocytes it is present as two subunits (S and I) with partially overlapping substrate specificities generated by extracellular proteolytic cleavage of a complex-glycosylated precursor (cP) [64,65]. In Caco-2 and HT-29 cells the cP is not cleaved into the two subunits after its insertion into the luminal membrane [4,5]. At least two intracellular high-mannose precursors have been identified [4,5]; the initial translation product (hmP_1), which is present in the endoplasmic reticulum and is very sensitive to proteolytic degradation [66], and an immunologically and conformationally distinct derivative (hmP_2) which is formed within 1 h of synthesis and is more resistant to proteolysis [66]. Evidence has been obtained recently suggesting the existence of a third form of high-mannose precursor, which has been localized in the intermediate or salvage compartment or in the Golgi complex [66].

To investigate the regulatory mechanisms responsible for the marked differences in SI activity among Caco-2 cell clones, we have studied the progressive accumulation of the enzyme in the brush border membrane after formation of confluent monolayers, and its biosynthesis and intracellular processing in clones characterized by low, intermediate or high sucrase activity. Enzyme activity (Fig. 3) and amounts of immunoreactive protein (Figs. 4 and 5) increased in parallel and in a nearly linear fashion after the cells had reached confluence, although at quite different rates in low- and high-SI-producing clones. These results suggest that the difference in sucrase activity among Caco-2 cell clones was not due to differential expression of forms of SI endowed with low or absent enzyme activity, previously identified in human small intestinal [5] and colonic [21] crypt cells *in vivo*.

An approx. 2-fold difference was observed in SI mRNA levels between high- or intermediate- and low-SI-producing clones (Fig. 6 and Table 5) which paralleled the cells' ability to synthesize SI over a 1 h period (Table 4). However, these differences among clones could not entirely account for the corresponding differences in sucrase activity levels and amounts of immunoreactive SI present in the same cells, suggesting that post-translational processing of the enzyme also played an important role. This conclusion is also supported by the observation that Caco-2/5 and Caco-2/15 cells had very similar SI mRNA levels and synthesized equal amounts of SI, while

exhibiting a 2.5-fold difference in sucrase activity in their brush border membranes.

Further evidence for a post-translational regulation of SI expression was obtained in pulse–chase experiments comparing SI and AP synthesis in Caco-2/3 and Caco-2/15 cells (Figs. 7 and 8). The rates of conversion of the intracellular SI biosynthetic forms did not differ significantly among the two clones. However, the initial 2-fold difference in SI synthesis during the 1 h pulse increased to a 3–3.5-fold difference at the time of complete conversion of hmP_1 into hmP_2 + cP, and further increased to a 5–5.3 fold difference after complete conversion into cP. These findings seem to implicate intracellular degradation of newly synthesized SI, presumably taking place in the endoplasmic reticulum, Golgi complex [67] or lysosomes [14], as an important factor in the regulation of SI expression in intestinal cells.

It is well established that proteins which fail to fold properly or to undergo required conformational changes are retained in the endoplasmic reticulum and eventually destroyed there by one or more resident proteolytic enzymes [68]. Such a process may explain the decreased amounts of hmP_2 and cP formed in Caco-2/3 cells from the initially co-translationally glycosylated hmP_1 precursor, which is known to be particularly susceptible to proteolysis [66]. The faster degradation rate of cP in these cells may have been due to its diversion from the Golgi complex to lysosomes, a process which has been previously observed both in Caco-2 cells [14] and in intestinal cells *in vivo* [69]. Similarly, several forms of human SI deficiency have been attributed to blockage of SI transfer to the cell surface, leading to its accumulation in different intracellular compartments [67].

In conclusion, our results suggest the existence of different mechanisms regulating SI expression in intestinal cells: (*a*) some affecting cytoplasmic mRNA levels and possibly including transcriptional regulation, regulation of SI mRNA exit from the nucleus or its stability in the cytoplasm, likely to be responsible for the 2-fold difference in SI synthesis we have observed between low- and high-SI-producing clones; and (*b*) post-translational processing and/or intracellular degradation affecting one or more of the different SI biosynthetic forms. These processes may at least in part, be susceptible to regulation by growth factors synthesized and secreted by the same cells into the culture medium.

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