Lactase and sucrase–isomaltase gene expression during Caco-2 cell differentiation

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The Caco-2 cell line is derived from a human colon adenocarcinoma and differentiates *in vitro* into small-intestinal enterocyte-like cells, expressing the hydrolases lactase and sucrase–isomaltase. We cultured Caco-2 cells on permeable supports from 0 to 37 days after plating to study endogenous lactase and sucrase–isomaltase gene expression in relation to cell differentiation. Profiles of lactase and sucrase–isomaltase mRNA, protein and enzyme activity were analysed on a per-cell basis, using immunocytochemistry, RNase protection assays, metabolic polypeptide labelling and enzyme activity assays. Tight-junction formation was complete 6 days after plating. Immunocytochemistry of Caco-2 cross-sections showed lactase and sucrase–isomaltase predominantly in the microvillar membrane of polarized cells. mRNA, protein and enzyme activity of lactase appeared consecutively, reaching maximum levels 8–11 days after plating. Whereas lactase mRNA and protein biosynthesis showed a sharp decline after peak levels, lactase activity remained high until 37 days after plating. In contrast, mRNA and protein biosynthesis and activity of sucrase–isomaltase peaked successively 11–21 days after plating, and exhibited comparable levels throughout the entire experiment. The following conclusions were reached. (1) In Caco-2 cells, biosynthesis of lactase and sucrase–isomaltase is regulated by the amount of their mRNAs, indicating transcriptional control. (2) Sucrase–isomaltase activity is most probably transcriptionally controlled at all time points. (3) In contrast, lactase activity is initially regulated by its level of biosynthesis. After its peak at 8 days, the slow decline in activity compared with its biosynthesis indicates high stability. (4) Different mRNA profiles for lactase and sucrase–isomaltase indicate different mechanisms of transcriptional regulation of these genes.

INTRODUCTION

The human colon adenocarcinoma cell-line Caco-2 provides one of the most relevant models for studying characteristics and mechanisms of epithelial cell differentiation *in vitro* such as polarization, microvillar membrane assembly and protein trafficking [1–4]. Caco-2 cells proliferate and differentiate on cell-to-cell contact into polarized enterocyte-like cells with a well-developed microvillar membrane [5,6]. Furthermore, Caco-2 cells represent the only cell line known to express endogenous lactase as well as sucrase–isomaltase [7]. We took advantage of this cell model to study the regulation of lactase and sucrase–isomaltase quantitatively per individual epithelial cell.

In human intestine, lactase is synthesized as a 200 kDa precursor containing high-mannose N-linked glycans, which is converted into a 217 kDa complexly N-glycosylated form before proteolytic cleavage, which yields a mature 160 kDa enzyme that is inserted into the microvillar membrane [8,9]. However, in Caco-2 cells there are minor differences from the situation *in vivo*, as both intracellular transport and processing of lactase into the mature microvillar membrane enzyme are relatively slow [7]. In human intestine, sucrase–isomaltase is synthesized as a 210 kDa precursor containing high-mannose N-linked glycans, which is converted into a 245 kDa complexly N-glycosylated form that is inserted into the microvillar membrane before proteolytic cleavage into its two enzymic subunits, sucrase (145 kDa) and isomaltase (151 kDa) [10]. Sucrase–isomaltase biosynthesis in Caco-2 cells only differs from that *in vivo* with respect to the absence of proteolytic cleavage, which normally occurs at the luminal side of the enterocyte by trypsin-like pancreases [7].

Many aspects of developmental expression patterns of lactase and sucrase–isomaltase have been studied [1]. *In vivo*, developmental enzyme activity profiles correlate well with the change in diet around the time of weaning from predominantly milk (containing lactose) to non-milk food containing other disaccharides (sucrose) and polysaccharides. Pronounced changes in expression of lactase and sucrase–isomaltase occur during this weaning period: lactase-specific activity declines about tenfold, whereas sucrase–isomaltase is not present in appreciable amounts until weaning, but increases soon thereafter to high levels and remains high throughout life [11,12]. Although co-varying to some extent with dietary carbohydrate levels, expression of lactase and sucrase–isomaltase is not primarily regulated by the presence of their substrates, but appears to be genetically determined [13,14]. Aspects of the temporal and spatial patterns of lactase and sucrase–isomaltase gene expression *in vivo* in several species (rat, rabbit, pig, human and mouse) have been described [15–24]. However, quantitative interpretation of these studies is hampered because tissue homogenates were used and consequently many cell types were studied simultaneously, thereby obscuring the contribution of enterocytes to the levels of disaccharidase mRNA, protein and activity. Moreover, changes during development in hormone levels, morphology of the intestine, and luminal factors, e.g. appearance of pancreatic proteases, further complicate the interpretation of regulation of gene expression *in vivo*.

At present, Caco-2 is the only human cell-culture model that might exhibit temporal, most probably differentiation-dependent, lactase and sucrase–isomaltase gene expression. We have chosen to study regulation of these genes in conjunction with

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Caco-2 cell differentiation by analysing mRNA, enzyme biosynthesis and enzyme activity relative to the number of cells. This enables us to determine the relative importance of each level of regulation for both enzymes during cytodifferentiation. The results show that both sucrase and lactase are primarily regulated by the amount of their mRNAs. However, the two enzymes display different characteristic mRNA profiles, indicating independent transcriptional regulation. Moreover, protein stability exerts additional regulation with respect to the levels of enzyme activity.

MATERIALS AND METHODS

Cell culture

Caco-2 cells were cultured in 75 cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium with 4.5 g/l glucose (Gibco/Life technologies, Breda, The Netherlands) supplemented with 0.1 mM each non-essential amino acid, 10 % foetal calf serum, 50 m-units/ml penicillin (Sigma, St. Louis, MO, U.S.A.) and 50 μg/ml streptomycin (Sigma). Medium was replaced every 2–3 days. Cells were routinely treated with trypsin at near-confluent densities and split 1 to 10 every week for at least 4 weeks. Then for all experiments cells were seeded on permeable collagen-coated filters (Transwell-Col; Costar, Cambridge, MA, U.S.A.) between passage number 94 and 104 at high densities (2×10^5 cells/cm^2) with 2.5 ml of medium in the basal compartment and 1.5 ml in the upper compartment, which was replaced after 18 h to remove unattached cells. Thereafter, media in both compartments were replaced every 2–3 days. Cells were incubated in 5 % CO_2, at 95 % relative humidity and 37 °C.

Cell counting

Filters were rinsed once in PBS, pH 7.4, and fixed for 20 min at room temperature in methanol/acetic acid (3:1, v/v). They were then rinsed with water and incubated for 30 min at room temperature with 1 μg/ml bisbenzimide H 33258 (Calbiochem, La Jolla, CA, U.S.A.) in 0.1 M sodium acetate, pH 5.0. The number of nuclei per micrograph, representing a surface area of 0.06 mm^2, was determined by fluorescence microscopy and photography. The numbers of cells per filter (4.7 cm^2) was then calculated.

Specificity of the monoclonal antibodies

The monoclonal antibody directed against sucrase–isomaltase (HBB 2/219/20) recognized the protein precursor (210 kDa) as well as the complexly N-glycosylated form (217 kDa). As the sucrase–isomaltase precursor forms are not further processed in Caco-2 cells, only these forms were detected [7]. The monoclonal antibody directed against lactase (HBB 1/90/34/74) recognized the protein precursor (200 kDa), the complexly N-glycosylated (214 kDa) form and the proteolytically cleaved mature form (143 kDa) [7].

Trans-monolayer resistance

Trans-monolayer resistance was measured as a parameter for tight-junction assembly. The electrical potential difference generated by bidirectional 10 μA pulses across the monolayer was monitored by electrodes placed at both sides of the monolayer. Tight-junction formation was considered complete when electrical resistance exceeded 500 $\Omega \cdot cm^2$ compared with a filter without cells [25].

Immunocytochemistry

Filters with cells were rinsed with PBS, fixed for 30 min in 4 % paraformaldehyde in PBS, dehydrated in ethanol (50, 70, 80, 96 and 100 %), incubated overnight in butan-1-ol, embedded in paraffin, and stored at 4 °C until sectioned. Slides with 7 μm-thick sections were deparaffinated in xylene and hydrated in ethanol (100, 90, 80, 70, 50 %), rinsed in PBS and used either for routine haematoxylin/azofloxin staining or immunoperoxidase detection of lactase or sucrase–isomaltase essentially as described by Rings et al. [26].

RNase-protection assay

Cytoplasmic RNA was isolated using Nonidet P40 (BDH, Poole, Dorset, U.K.) [27]. RNA yield was calculated from the absorbance at 260 nm. The integrity and quantity of the RNA was further analysed by agarose-gel electrophoresis. In all experiments, 5 μg samples of cytoplasmic RNA were used for the RNase-protection assays. Templates for antisense RNA probes were synthesized as described by Krasinski et al. [28]. A template for human lactase mRNA, constructed in pIBI31 (International Biotechnologies, New Haven, CT, U.S.A), was linearized with *Hin*dIII before transcription. A template for human sucrase–isomaltase mRNA, constructed in pBS(KS$^-$) (Stratagene, La Jolla, CA, U.S.A.), was linearized with *Sal*I before transcription. Antisense RNA probes were synthesized by modifications of the method described by Sambrook et al. [27]. Linearized templates (0.1 μg) were incubated with transcription buffer containing 2.5 mM each ATP, UTP and GTP, 40 μmol of [α-^{32}P]CTP (800 Ci/mmol, Amersham, Bucks., U.K.), and 10 units of DNA-dependent RNA polymerase (Pharmacia, Uppsala, Sweden) in a final volume of 10 μl for 1 h at 37 °C. After treatment with RNase-free DNase I (Boehringer-Mannheim, Almere, The Netherlands) to digest the DNA template, non-incorporated ribonucleotides were removed on a Sephadex G-50 column (Pharmacia). Probe-containing fractions were pooled, precipitated in ethanol and solubilized in hybridization solution containing 50 % formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA and 0.1 % SDS. Specific radioactivities of the antisense probes were approx. 5×10^4 c.p.m./fmol. Antisense probes (5×10^4 c.p.m.) were added to the RNA sample, denatured for 5 min at 85 °C, and hybridized for 18 h at 68 °C. Non-hybridized RNA was digested with RNase A (20 μg/ml; Boehringer-Mannheim) and RNase T1 (2 μg/ml; Boehringer–Mannheim), and the protected fragments were run on a 6 % polyacrylamide/8 M urea gel with a resolution capacity of one nucleotide, together with end-labelled single-stranded DNA fragments of known size as markers. An approx. 300-fold excess of radiolabelled probe was hybridized with the complementary target mRNA isolated from Caco-2 cells. Gels were fixed for 10 min in 10 % acetic acid/10 % methanol and dried before autoradiography or quantification as described below.

Metabolic labelling and immunoprecipitation

Cell monolayers were washed once with 4 ml of PBS, and intracellular methionine was depleted through incubation in Eagle's minimal essential medium (Gibco), containing non-essential amino acids and penicillin/streptomycin, without methionine for 30 min under the above conditions. ^{35}S-labelled amino acids (100 μCi, Cell Labelling Mix; Amersham International, Amersham, Bucks., U.K.) containing [^{35}S]methionine (specific radioactivity > 1000 Ci/mmol) was added basally. Although this preparation also contains [^{35}S]cysteine, experiments were per-

formed in the presence of sufficient unlabelled cysteine to prevent significant incorporation of this relatively unstable amino acid. After 20 h of incubation, during which radiolabel incorporation was linear with time, filters were rinsed once in PBS, cut out of their frames and homogenized by 20 strokes in a Potter–Elvejhem tube in 2 ml of ice-cold 20 mM Hepes, containing 20 mM NaCl, 0.02% (w/v) NaN_3, 0.5% (v/v) Triton X-100 (BDH), 100 μg/ml soyabean trypsin inhibitor (Sigma), 10 μg/ml pepstatin A (Sigma), 10 μg/ml leupeptin (Sigma) and 1 mM PMSF (Sigma). The homogenate was divided into equal aliquots to which an excess of IgG was added of either anti-(human lactase) or anti-(human sucrase–isomaltase) monoclonal antibody. After 4 h of incubation at 4 °C, samples were cleared by centrifugation for 5 min at 10000 ***g***. The supernatant was incubated for 1 h at 4 °C with 50 μl of Protein A–Sepharose beads (Pharmacia) with sufficient capacity to bind the IgG. Immunocomplexes were washed once in 50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 0.05% Triton X-100 and 0.1% SDS, and twice in 50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 0.1% Triton X-100 and 0.02% SDS and protease inhibitors as described above. Protein A–Sepharose pellets were dried by aspiration, and boiled for 5 min in 40 μl of sample buffer (125 mM Tris/HCl, pH 6.8, 20% glycerol, 5% SDS, 2% 2-mercaptoethanol, 0.001% Bromophenol Blue) [29]. Each sample was analysed by SDS/PAGE (7.5% gel). Gels were stained with Coomassie Blue, incubated for 15 min in Amplify (Amersham International) and dried for fluorography and quantification as described below. The molecular masses of lactase and sucrase–isomaltase were compared with prestained molecular-mass standards (Bio-Rad, Veenendaal, The Netherlands). All samples were boiled and stored at −20 °C immediately after immunoprecipitation until electrophoresis. A single batch of [^{35}S]methionine was used at all time points of an experiment to circumvent correction for radioactive decay.

Assay of enzyme activity

Lactase and sucrase activities were determined in aliquots of total cell homogenates using the natural substrates lactose and sucrose [30]. To inhibit lysosomal acid β-galactosidase activity fully, we performed the assay in the presence of 1 mM *p*-chloromercuribenzoate [30].

Quantification of the results

Four independent series of Caco-2 differentiation experiments were performed, two covering 0–8 days and two covering 0–37 days after plating. Therefore in all graphs mean values ± S.E.M. from at least two independent experiments are shown. Data were corrected for cell number, and also for RNA yield/cell in the case of the RNase-protection assay. Before calculation of the mean using data from independent experiments, data from each experiment were indexed to generate values between 0 to 1, so that data from each experimental series would evenly contribute to the mean. The resulting profile of each technique was again indexed and plotted in each graph as arbitrary units between 0 and 1. RNase-protection assays as well as metabolic labelling experiments were analysed with a PhosphorImager using ImageQuant software (Molecular Dynamics) to determine the amount of radioactivity in each band. Band surface areas were of equal size at all time points. Protected riboprobe band intensities were corrected for background by subtracting the 'signal' in an equal surface area in a lane where yeast tRNA or human thyroid-gland RNA was used to hybridize to the respective probes. This corrected 'signal' was then divided by the number of cells per filter and multiplied by the yield of total RNA at that specific time point to give the amount of specific mRNA per cell. Band areas for lactase biosynthesis were chosen to include the 200, 214 and 143 kDa forms, whereas for sucrase–isomaltase the area included both the 210 and 217 kDa forms. All measurements were corrected for background by substracting the 'signal' from an equal surface area in a lane representing an immunoprecipitation from 1 ml of apical medium. The resulting 'signals' were then divided by the number of cells per filter to give the amounts of lactase and sucrase–isomaltase synthesized *de novo* per cell at each time point. Enzyme activities were corrected for number of cells per filter at each time point. As background values, we used the results from an assay with the same homogenate, which was incubated first with Tris, a fully competitive inhibitor of lactase as well as sucrase [30], before incubation with the substrate (lactose or sucrose).

RESULTS

Caco-2 cells were seeded on collagen-coated filters at very high near-confluent densities. After seeding, the number of cells, or more precisely the number of detected nuclei, was determined. Figure 1 shows a small increase in cell number over the first 6 days after seeding, and relatively unchanged cell numbers thereafter. As mitotic figures were virtually absent after 6 days, we conclude that cells ceased to proliferate after this amount of time in culture. Total RNA was isolated from cells at each time point, analysed by agarose-gel electrophoresis and its concentration determined by measuring the A_{260}. As the gel showed that all samples were comparable with respect to RNA integrity, the absorbance values were used to plot Figure 1. The total amount of RNA isolated per filter showed a very similar profile to that of cell numbers. Thus the ratio between cell number and amount of RNA remained similar throughout the experiments.

Polarization of the cells was measured as trans-monolayer resistance, which is indicative of tight-junction formation. Figure 2 shows that trans-monolayer resistance was not detectable until 4 days after seeding, but increased rapidly thereafter reaching a maximum at 7 days. In accordance with other studies, we conclude that above 500 $\Omega \cdot cm^2$, Caco-2 cells form a polarized confluent monolayer [25]. Polarization was further studied by immunocytochemistry of lactase and sucrase–isomaltase, which

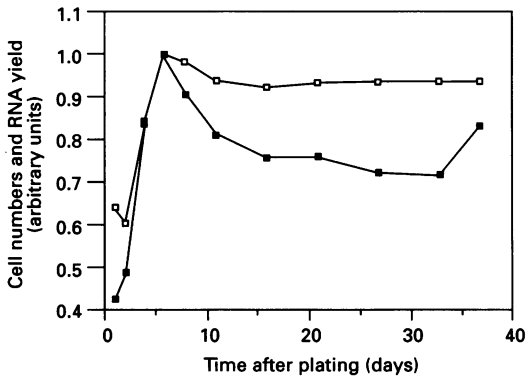

Figure 1 Cell number and RNA yield during Caco-2 cytodifferentiation

Cell number (□) and RNA yield (■) per filter were plotted in arbitrary units against time after plating of Caco-2 monolayers. Results are means of at least two experiments. Maximal cell density was 2.3×10^6 cells/filter at 6 days. Maximal total RNA yield was 95.2 μg/filter at 6 days.

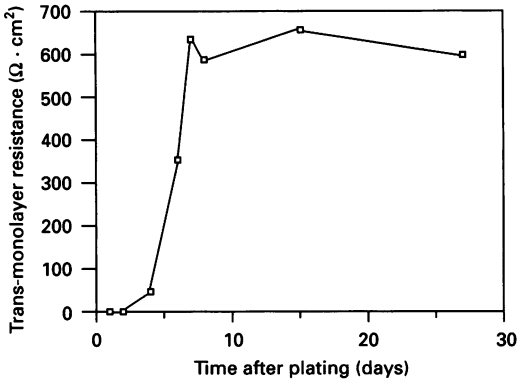

Figure 2 Trans-monolayer resistance during Caco-2 cytodifferentiation

Transwell filters with monolayers of Caco-2 cells were subjected to bidirectional 10 μA electrical pulses and the trans-monolayer resistance ($\Omega \cdot cm^2$) was plotted against time after plating.

are known to be localized exclusively in the microvillar membrane of polarized Caco-2 cells [7,31]. Figure 3 shows representative immunocytochemistry of lactase and sucrase–isomaltase at 8 and 16 days after plating respectively. Neither enzyme was detected immunocytochemically until 5 days, but the polypeptides were detected in cells from 7 days onwards. During early Caco-2 cytodifferentiation, expression of either of these polypeptides was first only found in patches of cells, but within a few days all cells were positive. Both polypeptides were expressed predominantly in the microvillar membrane, but some intracellular staining was also found in the region of the rough endoplasmic reticulum and Golgi complex. Neither lactase nor sucrase–isomaltase was detected at the basolateral plasma membrane of the cells. Negative controls, i.e. immunocytochemistry without lactase- or sucrase–isomaltase-specific antibodies, showed no staining at any time point.

Expression of lactase and sucrase–isomaltase genes was measured at the level of mRNA and protein biosynthesis and enzyme activity. Figures 4(a) and 4(b) show representative RNAase-protection assays detecting lactase and sucrase–isomaltase RNA respectively during differentiation of the cells. Lactase mRNA concentration clearly peaks at 6–8 days (Figure 4a). Four separate bands, each differing by only one nucleotide in size, were consistently found when the lactase antisense probe was used, and are most likely the result of incomplete RNA digestion. As these bands were characteristic of the use of this probe, all four were used to calculate the amount of lactase mRNA. Lactase mRNA content per cell increased linearly up to 8 days and rapidly decreased thereafter to low levels (Figure 4c). In one of the experiments the peak value fell at 6 days, hence the large S.E.M. at this time point. More convincing, however, is the decline in lactase mRNA per cell to 10–15% of its maximal value at 8 days. Sucrase–isomaltase mRNA steadily increased from 0 to 16 days (Figure 4c). Sucrase–isomaltase mRNA abundance reached maximal values at 11 days but, as can also be seen from the indicated S.E.M. bars, these peak levels were maintained until 16 days. Thereafter sucrase–isomaltase mRNA decreased reaching approx. 65% of its peak value at 37 days (Figures 4b and 4c). Negative controls, i.e. hybridization of either lactase or sucrase–isomaltase antisense probes with yeast tRNA and human thyroid RNA, showed no signals.

Biosyntheses of lactase and sucrase–isomaltase *de novo* was measured during Caco-2 cell differentiation. Lactase biosynthesis increased linearly, starting at 2 days and reaching a peak at 8 days (Figures 5a and 5c). After 8 days, it rapidly decreased to less than 10% of its peak level. Sucrase–isomaltase biosynthesis, like that of lactase, also occurred first at 2 days (Figure 5b), but, after a steady increase it reached a peak at 21 days (Figure 5c).

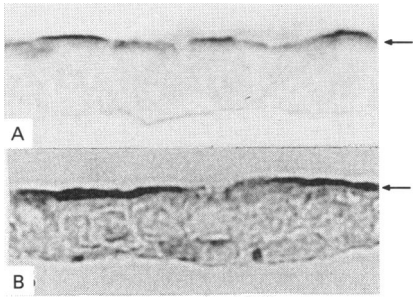

Figure 3 Immunocytochemical detection of lactase and sucrase–isomaltase in Caco-2 cells

(A) Lactase was detected in the apical membrane of Caco-2 cells 8 days after plating, using the HBB 1/90/34/74 monoclonal antibody. (B) Sucrase–isomaltase was detected in the apical membrane 16 days after plating, using the HBB 2/219/20 monoclonal antibody. The arrows indicate the apical membrane of the Caco-2 cells. During fixation, the Caco-2 cells usually became detached from the filter, and therefore the filter is absent from the micrographs.

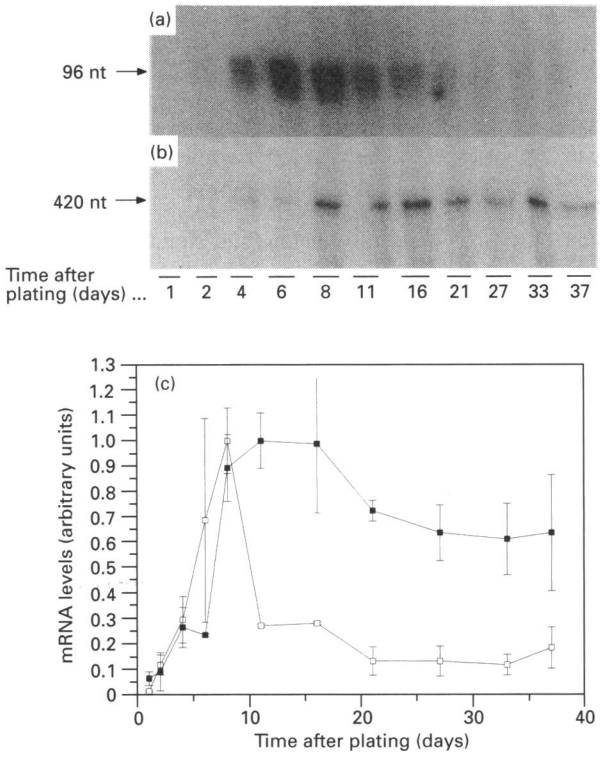

Figure 4 Lactase and sucrase–isomaltase mRNAs during Caco-2 cytodifferentiation

RNase-protection assays using [^{32}P]CTP-labelled riboprobes showed a protected fragment of 96 nucleotides representing lactase mRNA (**a**) and a protected fragment of 420 nucleotides representing sucrase–isomaltase mRNA (**b**) at the indicated times. Band intensities were calculated from two to four independent experiments using a PhosphorImager. Data were processed to generate the graph (**c**), as indicated in the Materials and methods section. □, Lactase; ■, sucrase–isomaltase.

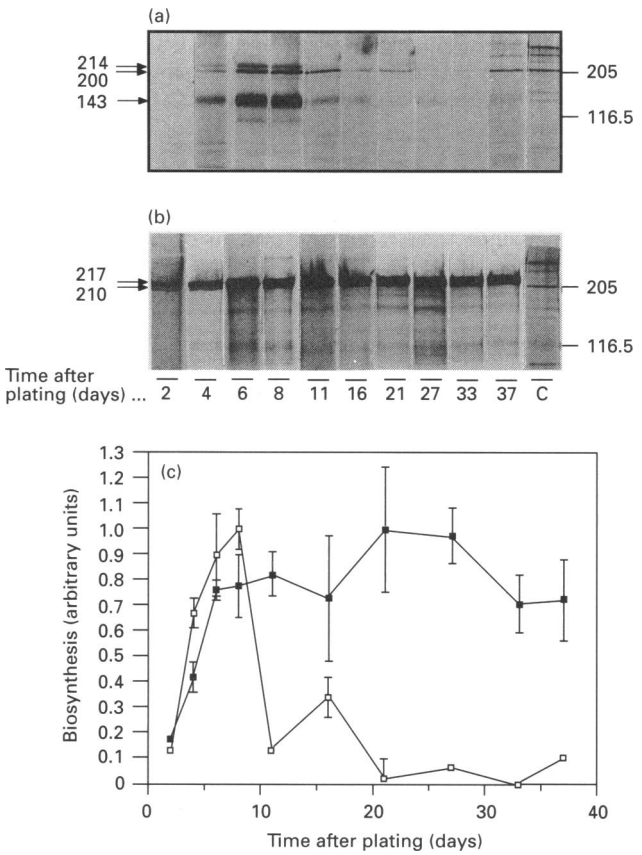

Figure 5 Biosynthesis of lactase and sucrase–isomaltase during Caco-2 cytodifferentiation

Caco-2 cells were labelled for 20 h at the indicated time with [^{35}S]methionine. Lactase (**a**) and sucrase–isomaltase (**b**) were immunoprecipitated, analysed by SDS/PAGE and fluorographed for 6 days. Molecular-mass markers (kDa) are indicated on the right. Lanes C represent control immunoprecipitations from the media of the apical compartment at 2 days. Band intensities from two to four independent experiments were determined using a PhosphorImager. Data were processed to generate the graph (**c**), as indicated in the Materials and methods section. Note that the labelling procedure did not allow a measurement at 1 day. This would require depletion and subsequent addition of [^{35}S]methionine immediately after cell plating, i.e. before cell attachment. □, Lactase; ■, sucrase–isomaltase.

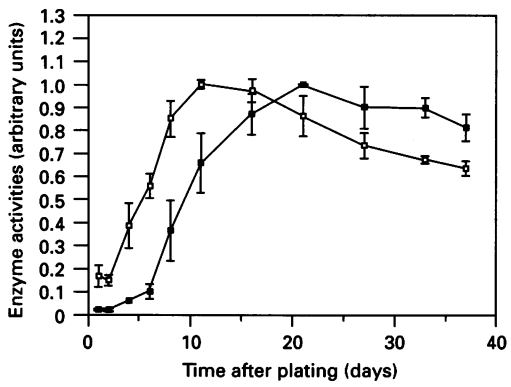

Figure 6 Lactase and sucrase activities during Caco-2 cytodifferentiation

The enzyme activities were measured using lactose and sucrose (each at 56 mM) respectively. The graph shows the mean of four independent experimental series. Data were processed as indicated in the Materials and methods section. The lactase peak activity found at 11 days corresponded to 6 m-units per filter and the sucrase peak at 21 days corresponded to 23 m-units per filter. Note that this is 3 m-units/10^6 cells for lactase and 11 m-units/10^6 cells for sucrase. □, Lactase; ■, sucrase–isomaltase.

Thereafter, it was decreased slightly to approx. 70% of it peak value. Figure 5(a) shows three molecular forms of lactase, 214, 200 and 143 kDa, as described by Hauri et al. [7]. This indicates that lactase was proteolytically cleaved to its mature microvillar membrane form at all time points. As previously noted [7], sucrase–isomaltase, in contrast with the situation *in vivo*, was not cleaved to its sucrase and isomaltase subunits, as only two molecular forms could be distinguished, 217 and 210 kDa (Figure 5b).

The disaccharidase activities in homogenates of Caco-2 cells were measured during cell differentiation (Figure 6). The lactase activity showed a linear increase starting at 2 days, reaching a peak at 11 days (6 m-units/filter). A subsequent slow decline was observed to about 65% of the peak activity at 37 days. Sucrase activity started to increase at 6 days, and reached its maximal value, after a gradual increase, at 21 days (23 m-units/filter); thereafter it decreased gradually towards 37 days, at which time it was still 85% of its maximal value. Similar enzyme assays were performed on microvillar membrane preparations after cell fractionation by the Ca^{2+}-precipitation method [32]. Essentially similar profiles were obtained to both hydrolases, but enzyme recovery was relatively low.

DISCUSSION

The intestinal cell line Caco-2 has been used to study various aspects of epithelial cytodifferentiation, such as barrier formation, trans-epithelial transport, expression of enterocyte-specific genes and routing of proteins synthesized *de novo* within the cell [3,7,25,33,34]. Recently, Hauri et al. [35] showed modulation of lactase and sucrase–isomaltase expression in Caco-2 cells 13–16 days after seeding, using cyclic AMP modulators. Vachon and Beaulieu [36] have described the endogenous expression of sucrase–isomaltase during Caco-2 cell differentiation at the immunocytochemical level. The present study is the first to analyse lactase and sucrase–isomaltase at the mRNA, protein biosynthesis and enzyme activity levels as well as cell numbers during an extended period. The major advantage of Caco-2 cells over tissue is that the results can be expressed on a per-cell basis, thereby greatly facilitating interpretation of the regulation of these genes at the cellular level.

Immunocytochemical analysis showed, as expected and similar to the situation *in vivo*, predominant microvillar localization of both glycohydrolases. It also showed that not all cells were stained during the upsurge in lactase and sucrase–isomaltase biosynthesis, showing a patchy pattern of expression. Similar findings in Caco-2 monolayers have been reported by Vachon and Beaulieu [36] for sucrase–isomaltase and by Hauri et al. [7] for lactase. As all differentiating Caco-2 cells eventually express lactase and sucrase–isomaltase within a few days of their initial detection, the patchy expression of the glycohydrolases appears to be a transient phenomenon and probably results from slightly asynchronous differentiation of adjacent cells [36]. As this phenomenon exists for only a short period compared with the span of the experiment, it does not seriously interfere with the interpretation of the measurements.

In vivo, patchy expression of lactase was shown in rat and human and probably results from a different mechanism, as discussed above for Caco-2 cells [16,37]. Yet, no explanation was found for this pattern of gene expression. *In vivo*, the intestinal epithelium is continuously repopulated by descendants of stem

cells residing in the crypt. The differentiation of epithelial cells correlates closely with their position along the crypt–villus axis. It is generally accepted that villus enterocytes are fully differentiated cells. In patchy lactase expression, the ratio between lactase-positive and -negative enterocytes along the villus is unchanged, indicating that lactase expression is not correlated with cell age [16,37]. Therefore the eventual expression of lactase of each villus enterocyte is probably determined earlier during a specific stage of differentiation in the crypt.

Insight into the regulation of lactase and sucrase–isomaltase expression in Caco-2 cells can be acquired by analysing the profiles of mRNA and protein biosynthesis and activity of each enzyme. Lactase mRNA levels were closely correlated with lactase biosynthesis at all time points (Figures 4c and 5c), indicating that biosynthesis is primarily regulated at the transcriptional level. Lactase activity occurs about 2 days after the peak in biosynthesis, but declines only very slowly to about 70% of its peak value at the end of the experiment (Figures 5c and 6). This indicates that a high stability of lactase determines the enzyme levels after 11 days, when the biosynthesis is reduced to low levels. Sucrase–isomaltase mRNA levels were also correlated closely with sucrase–isomaltase biosynthesis, indicating primary regulation at the transcriptional level (Figures 4c and 5c). At early (up to 8 days) and later (after 21 days) time points, sucrase–isomaltase biosynthesis is high, relative to the content of its mRNA, suggesting additional translational regulation. However, this translational regulation imposed only minor effects on the translation efficiency, as the biosynthesis levels vary only by about 20% relative to the mRNA levels (Figures 4c and 5c). Sucrase activity is closely correlated with the levels of sucrase–isomaltase biosynthesis. As there is no accumulation over time of active sucrase, the sucrase–isomaltase protein apparently has a short half-life. The profiles of sucrase–isomaltase activity, its biosynthesis and to a large extent also its mRNA levels are all correlated. Therefore the enzyme is probably transcriptionally regulated at all time points.

In Caco-2 cells, the biosynthesis of both lactase and sucrase–isomaltase is probably primarily regulated at the transcriptional level. Comparable results obtained *in vivo* were reported, with regard to human and rat small intestine [16,22,26,28,38,39]. However, the mRNA-expression profiles for the two glycohydrolases in Caco-2 cells, which are indicative of transcriptional regulation, are markedly different. Lactase mRNA appeared before sucrase–isomaltase mRNA, peaked early, and rapidly declined thereafter. Sucrase–isomaltase mRNA appeared more gradually, peaked about a week after lactase mRNA, and declined to a stable level of about 70% of its peak value. Therefore we conclude that lactase and sucrase–isomaltase mRNA levels are regulated differently during Caco-2 differentiation. This suggests that the regulatory mechanisms involved in the expression of these glycohydrolases are different. Several other studies have found evidence for regulatory mechanisms that modulate lactase and sucrase–isomaltase expression patterns. Hauri et al. [35] have indicated that the biosynthesis of these two enzymes in Caco-2 cells is reciprocally influenced by cyclic AMP modulators such as forskolin and vasoactive intestinal peptide, also suggesting different regulatory mechanisms for these genes. Troelsen et al. [40] have identified a transcription factor in pig small-intestinal enterocytes, NF-LPH-1, which was also demonstrated to be present in Caco-2 cells. These authors have further shown that this factor recognizes the same nucleotide sequence as the human sucrase transcription factor SIF1-BP, identified by Traber et al. [41], and therefore probably binds to the same promoter element, suggesting a common mechanism of regulation of the two genes [42]. However, our results show that neither the up-regulation nor down-regulation of lactase coincides with that of sucrase–isomaltase, and therefore, in addition to at least one common regulatory factor, other specific factors must be involved to explain their differential expression in Caco-2 cells.

The second major determinant of lactase and sucrase–isomaltase levels in Caco-2 cells, apart from their mRNA levels, is their stability. Lactase has a long half-life compared with sucrase–isomaltase. This could be explained by differences in processing of these enzymes during the span of the experiment. Proteolytic processing of lactase occurs during all stages of cytodifferentiation, because the mature form as well as the high-mannose and complexly N-glycosylated precursor were detected at all time points. Sucrase–isomaltase is not proteolytically cleaved in Caco-2 cells at any time point. Hauri et al. [7] reported similar findings for lactase and sucrase–isomaltase in Caco-2 cells at 5 and 9 days after confluence. Proteolytic processing of either enzyme appeared not to affect the enzyme activity [43–45]. As biosynthesis of both hydrolases shows similar patterns at different time points, it seems unlikely that changes in processing contribute to the observed changes in enzyme activity. Glycosylation changes during Caco-2-cell differentiation [46] are not likely to explain differences in half-lives either, for two reasons. First, changes in terminal glycosylation during rat postnatal development, from predominantly sialic acid to fucose residues, proved irrelevant to the lactase specific activity [15]. Secondly, it is unlikely that these changes in glycosylation have opposite effects on half-lives of sucrase–isomaltase and lactase. Dudley et al. [47] have reported similar mean resident times for lactase (7.8 h) and sucrase–isomaltase (5.8 h) in rat intestinal brush borders. However, these results were obtained in intestinal epithelium of intact rats, which is normally exposed to pancreatic enzymes. Thus the half-life is determined *in vivo* by three parameters: *de novo* biosynthesis, intrinsic stability of enzymes in the brush border and degradation by luminal proteases. As there were no proteases present during our experiments with Caco-2 cells, and the *de novo* biosynthesis was analysed at all time points, we conclude that the difference in stability of lactase and sucrase–isomaltase reflects intrinsic properties of these enzymes in Caco-2 cells.

The results of this study show independent expression of lactase and sucrase–isomaltase in Caco-2 cells. Therefore the differentiation-dependent characteristic of these two glycohydrolases may be used to investigate further at the cellular level transcriptional factors that govern lactase and sucrase–isomaltase expression.

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