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The human intestinal cell lines Caco-2 and LS174T as models to study cell-type specific mucin expression

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Mucin expression was studied during proliferation and differentiation of the enterocyte-like Caco-2 and goblet cell-like LS174T cell lines. Caco-2 cells express mRNAs of MUC1, MUC3, MUC4 and MUC5A/C whereas MUC2 and MUC6 mRNAs are virtually absent. Furthermore, MUC3 mRNA is expressed in a differentiation dependent manner, as is the case for enterocytes. Concomitantly MUC3 protein precursor (~550 kDa) was detected in Caco-2 cells. In LS174T cells mucin mRNAs of MUC1, MUC2 and MUC6 are constitutively expressed at high levels, whereas MUC3, MUC4 and MUC5A/C mRNAs are present at low levels. At the protein level LS174T cells express the goblet cell specific mucin protein precursors MUC2, MUC5A/C and MUC6 with apparent molecular masses of about 600 kDa, 470/500 kDa and 400 kDa respectively. MUC3 protein is not detectable. Furthermore, human gallbladder mucin protein (~470 kDa precursor), of which the gene has not yet been identified, is expressed in LS174T cells. In addition, synthesis and secretion of the goblet cell specific mature MUC2, MUC5A/C and human gallbladder mucin was demonstrated in LS174T cells. It is concluded that Caco-2 and LS174T cell lines provide excellent *in vitro* models to elucidate the cell-type specific mechanisms responsible for mucin expression.

Keywords: mucin-type glycoproteins, MUC1–6 mRNA biosynthesis, protein biosynthesis, Caco-2, LS174T, human intestine

Abbreviations: SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium; Endo-H, endo-β-*N*-acetylglucosaminidase H, HGBM, human gallbladder mucin; dpc, days past confluence; PBS, phosphate buffered saline.

Introduction

The gastrointestinal epithelium and other mucosa are covered by mucus. The mucus glycoproteins (mucins) are the most important structural components of mucus [1]. Mucins play an important role in cytoprotection. Alterations in intestinal mucins, especially in glycosylation patterns and expression levels, are thought to be associated with diseases such as inflammatory bowel disease and carcinoma [2–4]. Mucins are large glycoproteins and are either secretory or membrane-bound (reviewed in [5, 6]). The central part of the polypeptide backbone of epithelial mucins consists of many tandemly repeated amino acid sequences. These tandem repeats are rich in serine and threonine residues and may vary in number between individuals, but are invariably highly O-glycosylated. The C- and N-termini protrude from the heavily O-glycosylated central part, do not contain tandem repeats, and, for secretory mucins, contain many cysteine residues forming inter- and intramolecular disulfide bonds. Usually the termini have some N-glycosylation [6].

Due to diverse O-glycosylation, each mucin gene product eventually forms a heterogeneous set of molecules. Several different human epithelial mucin genes have been identified, named *MUC*1–8 [5]. MUC1, of which the gene has been fully sequenced, codes for a membrane-bound mucin [7–9]; MUC2 and MUC7 cDNAs have been completely sequenced and code for secretory mucins [10–12]. Of MUC3, 4, 5A/C, 5B, 6 and 8 only partial cDNA sequences are known [13–19]. Whether the latter cDNAs encode secretory mucins has

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to await their full sequence determination and cell biological measurement of the secretory status of each of these mucins.

Mucin expression is tissue- as well as cell-type specific and varies according to differentiation. For instance in the small intestine, MUC2 and MUC3 mRNA are prominently expressed: MUC2 is confined to goblet cells while MUC3 is largely confined to enterocytes, although some MUC3 expression is detectable in goblet cells [20]. In the human large intestine MUC2 is the prominent mucin [21] and is goblet cell specific, whereas in the stomach, which also produces a copious mucin layer, it is hardly detectable [2, 22]. In addition to MUC2 and MUC3, several other mucins, namely MUC1, MUC4, MUC5A/C, MUC5B and MUC6, have been reported to be expressed to some extent in the intestine [5]. Of the other known mucins MUC8 is not expressed in the small intestine as detected by Northern blot analysis and MUC7 expression seems restricted to the salivary glands [12, 17]. Besides the mucins of which the genes have been identified (*MUC*1–8) intestinal expression of at least one other mucin has been reported [23]. This mucin, human gallbladder mucin (HGBM), was originally identified in the human gallbladder, where it is prominently expressed and secreted [24]. In summary, MUC1–6 and HGBM are expressed in the intestine of which MUC2 and MUC3 predominate in the small intestine and MUC2 predominates in the large intestine.

In the intestine, besides the tissue- and cell-type specific expression, the differentiation status of small intestinal epithelial cells correlates closely with their position along the crypt-villus axis. In the small intestine MUC2 mRNA expression is detected along the whole crypt-villus axis; whereas MUC3 mRNA expression shows a gradient along the crypt-villus axis, with high expression on the villus tips [20]. In contrast, MUC1 expression is turned off early in differentiation when crypt cells differentiate and migrate up the villi [25]. Therefore, the expression of each mucin in the intestine seems to be regulated specifically during differentiation, but how this expression is regulated is still unknown.

Cell lines constitute good *in vitro* models to investigate the tissue- and cell-type specific mucin expression during proliferation and differentiation. In addition, these relatively homogeneous populations of cells allows one to investigate the regulation of cell-type specific mucin gene expression. To be able to understand cell-type specific mucin expression in the intestine we have characterized the mucin expression of two different human colon adenocarcinoma cell lines, LS174T and Caco-2. LS174T cells contain mucous granules, produce significant amounts of secretory mucin and have similarity to goblet cells [26, 27], while Caco-2 is a well differentiated enterocyte-like cell line developing a brush border [28–30]. After confluency Caco-2 differentiates into enterocyte-like cells, expressing i.e. the small intestinal glycohydrolases lactase and sucrase-isomaltase in an *in vitro* differentiation dependent fashion [30, 31]. The aim of this study was to characterize these two cell line models, for enterocytes and goblet cells, with respect to mucin biosynthesis during *in vitro* proliferation and differentiation. Mucin expression in LS174T and Caco-2 cells was therefore investigated at mRNA and protein level at various stages of cyto-differentiation.

Materials and methods

Chemicals were obtained from the following manufacturers: Amersham International, Amersham, Bucks., UK; Gibco/Life Technologies, Breda, The Netherlands; New England Biolabs, Beverly, MA, USA; Merck, Darmstadt, Germany; Sigma Chemical Co., St Louis, MO, USA; BioRad, Richmond, CA, USA; Pharmacia, Upsala, Sweden; BDH, Poole, Great-Britain and Boehringer Mannheim, Mannheim, Germany; ICN, Costa Mesa, CA, USA.

Cell culture

The LS174T cell line was obtained from American Type Culture Collection. The Caco-2 cell line was donated by Dr W.J. Van't Hoff, University of Utrecht. LS174T and Caco-2 were cultured in 4 cm^2 tissue culture wells and 25 cm^2 tissue culture flasks respectively (Costar, MA, USA) in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 4.5 $g\,l^{-1}$ glucose, 3.7 $g\,l^{-1}$ $NaHCO_3$, supplemented with 0.1 mM non-essential amino acids (Gibco), 50 $U\,ml^{-1}$ penicillin (Sigma), 50 $\mu g\,ml^{-1}$ streptomycin (Sigma). In addition the medium was supplemented with 20% and 10% fetal calf serum (Gibco) for LS174T and Caco-2 cells respectively. Cells were cultured in 5% CO_2 at 95% relative humidity and 37 °C. Medium was replaced daily for LS174T and once every 2–3 days for Caco-2. Cells were routinely treated with trypsin at near-confluent densities and split 1:2 (LS174T) or 1:4 (Caco-2). For experiments, LS174T and Caco-2 cultures were used between passages 112–122 and 95–102 respectively. Medium of post-confluent LS174T cells was centrifuged at 800 rpm to collect all living cells growing in suspension; these were reintroduced into fresh medium. Furthermore, for RNA experiments cells from one single passage of LS174T or Caco-2 were used and cells were seeded at identical densities in a set of wells or flasks, respectively, in order to isolate RNA at various days past confluency (dpc).

RNA blotting and quantification

MUC1 (450 bp), MUC2 (900 bp) and MUC3 (400 bp) cDNA sequences, cloned in the *Eco* RI site of pBluescript (Stratagene), have been described previously [7–10, 13]. All cDNA probes, except MUC5A/C contain tandemly repeated sequences. A MUC4 (48 bp) sequence was

obtained by standard polymerase chain reaction procedure [32] using MUC4 gene specific primers (see below) and an antisense MUC4 oligonucleotide as template. This template was kindly provided by Dr H. Vos and is based on the sequence 5′GTCGGTGACAGGAAGAGGGGTGG-CGTGACCTGTGGATGCTGAGGAAGT3′ [16]. MUC6 cDNA probe was also obtained by polymerase chain reaction using MUC6 gene-specific primers (see below) and human genomic DNA as template. Following is a list of oligonucleotides in which the engineered *Eco* RI (GSP-1) and *Sal* I (GSP-2) restriction sites are underlined.

MUC4 GSP-1: 5′GGAATTCGTCGGTGACAG3′
MUC4 GSP-2: 5′CTCTGTCGACTTCCTCAG3′
MUC6 GSP-1: 5′GGAATTCTGGAGTCACCAAGGT-GGAGAAAGGTGG3′
MUC6 GSP-2: 5′CTCTGTCGACACCACCTCAGCCA-CCAGCAGCAG3′

After amplification MUC4 and MUC6 DNA fragments were digested with *Eco* RI and *Sal* I and separated on a 2.5% agarose gel by standard procedures [32]. Subsequently, the DNA fragments were isolated from the gel using Qiaex gel extraction kit (Qiagen, Chatsworth, CA, USA) as described by the manufacturer, and cloned in the corresponding sites of pBluescript (Stratagene). MUC5A/C is described by Klomp and coworkers [33]. Sequences were verified using dideoxunucleotide sequencing method (USB, Cleveland, OH, USA) as described by the manufacturer. All mucin cDNA probes used for dot blot analysis were obtained by digestion of the appropriate restriction enzymes, followed by separation on agarose gels by standard procedure [32]. The cDNA probes were isolated using the Qiaex gel extraction kit, and labelled with α-[^{32}P]-dATP by the random prime labelling procedure [32]. Mouse β-actin antisense RNA probe (Ambion, Austin, TX, USA) was isolated and labelled as described by Krasinski and coworkers [34]. β-actin is highly conserved in higher eukaryotes [35].

Cytoplasmic RNA of cell lines was obtained by lysing cells with Nonidet P40 (BDH) followed by RNA extraction as described [32]. RNA was extracted in two independent series of experiments at preconfluency, 0, 3 and 5 days past confluency (dpc) for LS174T and at 0, 3, 5, 7, 10, 15, 20 and 25 dpc for Caco-2. Tissue RNA was obtained as follows: segments of human gallbladder, small and large intestine were obtained by operative procedure and the mucosa of non-diseased tissue was scraped and homogenized in guanidine isothiocyanate (GITC). Additionally, GITC lysate of a primary human tracheal cell culture was kindly donated by Dr P. Nettesheim. RNA was isolated from the GITC homogenates using centrifugation on a cesium chloride cushion as described [32]. Integrity of all RNAs was assessed by analysis of 5 μg RNA from cell lines and 10 μg RNA from tissue on 0.8% agarose gels followed by ethidium bromide staining to determine the intactness of the 28S and 18S rRNA bands. For RNA quantification two different quantities of RNA from cell lines (1 and 5 μg) at different confluency stages and of RNA from tissue (0.1 and 1 μg) was dot blotted onto N-Hybond membranes (Amersham) by a vacuum-operated dot blot apparatus (BioRad) followed by baking at 80 °C for 2 h. Hybridization of MUC1–6 probes and washings were performed as described [36]. Each probe was hybridized to all RNA samples simultaneously (RNA from cell lines and tissue) under the same conditions, making comparison between RNA derived from the two cell lines and tissue possible. Membranes were analysed with a PhosphorImager using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA, USA) to determine the amount of radioactive signal. The membranes were then washed to remove mucin probes, checked for residual radioactivity and hybridized to β-actin probe as a measure of the amount of RNA blotted. The MUC1–6 radioactive signals were expressed as a ratio relative to the β-actin signal to standardize for the amount of RNA. The values obtained from two series of experiments were averaged and a standard deviation was calculated. The same cDNA probes and hybridization conditions were used simultaneously for dot blot analyses of RNA from the different tissues and cell-lines. Therefore, expression patterns for each of the mucin mRNAs in the cell lines is directly and quantitatively comparable to the expression in the different tissues. In contrast, comparison between the expression patterns of the different mucins is much more difficult, because not all cDNA probes correspond to tandemly repeated regions of the mucin cDNA, i.e. MUC5A/C, and because of different probe labelling efficiencies and affinities. Thus, quantitative differences between each of the mucin mRNA levels can only be roughly estimated. The use of human tissue was approved by the medical ethical committee of our institution.

Antibodies

Rabbit polyclonal antisera were used, except for the mouse monoclonal WE9 which recognizes a peptide epitope in the unique termini of human MUC2 [37, 38]. Anti-HCM was raised against purified human colonic mucin and anti-RCM was raised against purified rat colonic mucin. Both these antisera recognize the unique non-O-glycosylated termini of rat as well as human MUC2 [21, 39]. WE9, anti-HCM and anti-RCM were therefore referred to as anti-MUC2 antisera. Anti-HGBM was raised against purified HGBM and recognizes predominantly unique non-O-glycosylated termini of HGBM [24, 37]. Anti-RGM was raised against purified rat gastric mucin and recognizes the unique termini of human and rat gastric mucin [40, 41]. By peptide sequencing the human gastric mucin was identified as MUC5A/C [33], therefore the anti-RGM represents an anti-MUC5A/C antiserum. Anti-

M6.1 was raised against a synthetic peptide representing the tandem repeat of MUC6 and affinity purified [42]. Anti-M3P was raised against a synthetic peptide representing the tandem repeat of MUC3 [2].

Metabolic labelling and immunoprecipitation

Mucin biosynthesis was studied by metabolic labelling experiments at time points of highest mRNA levels, i.e. in LS174T cells around confluency and in Caco-2 cells around 7 dpc. Cell monolayers were washed twice with phosphate buffered saline (PBS). Intracellular methionine/cysteine, threonine, or sulfate was depleted through incubation of 45 min in Eagle's minimum essential medium (EMEM, Gibco) containing 4.5 $g\,l^{-1}$ glucose, non-essential amino acids, penicillin and streptomycin, but without either methionine/cysteine or threonine or sulfate. Cultures were grown at 5% CO_2:95% O_2 at 37 °C and at high relative humidity.

Pulse labelling was performed for 30 min to assure labelling of precursor protein only. LS174T cells of different dpc were pulse labelled by adding either 20 μCi ^{35}S-labelled amino acids (Cell Labeling Mix, Amersham, specific activity 1000 Ci $mmol^{-1}$, containing 65% L-[^{35}S]methionine and 25% L-[^{35}S]cysteine) per tissue culture well to label the polypeptide or [^{35}S]sulfate (specific activity 1050 Ci $mmol^{-1}$, Amersham) to label mature sulfated glycoprotein [44]. Pulse labelling was performed at 20 μCi label per 200 μl medium per well. Chase incubations were performed for 4 h for sulfate labelling or 20 h for [^{35}S]methionine/cysteine labelling by washing the cell monolayer with PBS and then adding 2 ml unlabelled complete DMEM per tissue culture well.

Caco-2 cells were depleted in EMEM devoid of threonine (Gibco). Caco-2 cells were then pulse labelled for 45 min by adding 200 μCi of [^{3}H]threonine (ICN, specific activity 45 Ci $mmol^{-1}$). Pulse labelling was performed at 200 μCi per ml medium per flask.

After the respective pulse or chase experiments, cell monolayers were washed with PBS and homogenized at 4 °C in buffer containing 50 mM Tris, pH 7.5, 5 mM ethylenediaminetetraacetic acid, 1% (w/v) Triton X-100 (BDH), 1 mM phenylmethanesulphonyl fluoride (Sigma), 100 $\mu g\,ml^{-1}$ pepstatin A (Sigma), 100 $\mu g\,ml^{-1}$ leupeptin (Sigma), 10 mM iodacetamide (Sigma) and 0.24 U ml^{-1} aprotinin (Sigma). Mucin was immunoprecipitated from the homogenates overnight at 4 °C with anti-HCM, WE9, anti-RCM, anti-RGM, anti-M3P, anti-HGBM or anti-M6.1 antibodies. Immunocomplexes were precipitated using Sepharose CL-4B-coupled protein A (Pharmacia). Immunoprecipitated mucins were spun down and washed three times with buffer containing 1% (w/v) Triton X-100, 1% (w/v) sodium dodecylsulfate (SDS), 0.5% (w/v) sodium deoxycholate, 1% (w/v) bovine serum albumin (Boehringer) in PBS, followed by washing once in PBS diluted 10-fold. Samples were reduced with 2-mercaptoethanol and analysed on polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS using a 3% stacking and 4% running gel [43]. To analyse the mucin precursors, immunoprecipitated samples were treated with 750 U endo-β-*N*-acetylglucosaminidase (endo H) [21, 44]. For molecular weight markers, unreduced rat gastric mucin precursors, labelled with [^{35}S]amino acids, were used with molecular masses of about 300 kDa for the monomer and 600 kDa for the dimer [40]. Prestained high molecular weight markers, with molecular masses between 49.5 and 205 kDa (BioRad) were also used. Gels were fixed in 10% methanol:10% acetic acid, and incubated for 10 min with Amplify (Amersham). Radiolabelled mucins were analysed by fluorography, the film (Fuji, Japan) was exposed from 1 week to 3 months at −70 °C.

Results and discussion

MUC1–6 mRNA expression in tissue and cell lines

The aim of this study was to develop *in vitro* cell-type specific differentiation models of mucin expression comparable to the human intestine. Two cell lines, LS174T and Caco-2, and a variety of human tissues including small and large intestine, were examined with respect to mucin RNA and protein expression. RNA was isolated from cell lines at different time points during *in vitro* differentiation. The level of each mucin mRNA in tissue and cell lines was expressed as a ratio relative to the level of β-actin mRNA (Fig. 1). Levels of MUC4, MUC5A/C and MUC6 mRNA are relatively low in all tested tissues compared to MUC1–3 mRNAs and this may generally explain the large standard deviations in MUC4–6 mRNAs measurements in the expression profiles of the latter three mucin mRNAs in both cell lines.

From Fig. 1 it is clear that the mucins MUC1–6 mRNAs are expressed in a tissue specific manner, as was reported by others [2, 7, 10, 11, 19, 42, 45]. MUC1 mRNA is expressed in all tissues tested, which is in agreement with previously reported findings showing that MUC1 mRNA and protein are detectable in almost all human epithelia [2, 5, 45]. Primary tracheal cells in culture expressed highest levels of MUC1 mRNA, however MUC1 mRNA was expressed at relatively high levels in all tissues examined (Fig. 1). MUC2 mRNA expression is prominent in intestinal tissue, with highest levels in the large intestine, which correlates with the finding that MUC2 is the major mucin in the colon [21]. Furthermore, the high expression of MUC2 mRNA in large and small intestine has been shown previously [2, 10, 22, 45]. In contrast, in all other tissues analysed MUC2 mRNA expression was virtually absent. MUC3 mRNA, which was initially identified as an intestinal mucin [13], was expressed at highest levels in the small

intestine as would be expected, but also at high levels in the gallbladder and at low levels in stomach and large intestine (Fig. 1). The expression of MUC3 mRNA in gallbladder is in agreement with the finding that MUC3 protein could be isolated from human bile [46]. MUC4 and MUC5A/C were originally cloned from a tracheobronchial cDNA library [14, 16] and in agreement with this our results show that MUC4 mRNA was expressed at highest level in primary tracheal cells, while MUC5A/C mRNA was also expressed (Fig. 1). However MUC5A/C mRNA expression was highest in stomach, as was MUC6 mRNA of which the cDNA was cloned from a gastric tissue cDNA library [19]. MUC5A/C was recently identified as the major mucin in the stomach, and the high expression of MUC5A/C and MUC6 mRNAs in the stomach has been well documented [19, 41, 47]. MUC5A/C mRNA was expressed at very low levels in small and large intestine, gallbladder and primary tracheal cells. MUC6 mRNA was also expressed at low levels in gallbladder (Fig. 1). We detected hardly any MUC6 mRNA in small and large intestine. In summary, the expression of MUC1–6 mRNAs found in tissues in this study agrees with previously performed studies of ourselves and others and provide the proper positive controls for the use of our MUC1–6 cDNA probes in the analysis of cell lines. Furthermore, in order to develop a model for the intestine, the levels of MUC1–6 mRNA expressed in intestine can be related directly to those of LS174T and Caco-2 cells.

LS174T has goblet cell-like characteristics and accordingly was shown to synthesize several mucins of which the secretory MUC2 was most prominently expressed; while MUC1, MUC3 and MUC4 mRNAs were also detected [26, 27, 48, 49, 50–52]. We analysed the expression of MUC1–6 mRNAs from preconfluency until 5 dpc and compared this with the expression in tissue, especially the intestine. Generally LS174T cells detached from the tissue culture wells 7 days after reaching confluence. Likely, the LS174T cells had died as no RNA could be isolated from the cells and the cells had stopped producing metabolites. In LS174T cells MUC1, MUC3, MUC4 and MUC5A/C were expressed at levels about similar to that in the colon (Fig. 1). In addition, MUC2 mRNA was expressed at a level of about 20% of that found in colon, which is high considering that MUC2 is the prominent mucin in the colon. MUC6 mRNA was expressed in LS174T at a level similar to that in stomach, whereas MUC6 mRNA is not expressed in the colon. In summary, based on these results the human colonic cell line LS174T mimics the mucin expression in the colon, except for MUC6 mRNA expression. The expression of MUC6 mRNA indicates that LS174T cells have acquired gastric goblet cell-like characteristics, as these cells are known to express MUC6 [42, 19].

Interestingly, in LS174T MUC1–6 mRNAs have reached maximal expression levels already at preconfluence and remained at about stable levels thereafter. The early expression of MUC2 mRNA in undifferentiated LS174T cells at preconfluency correlates with the situation in the human intestine where MUC2 mRNA expression in intestinal goblet cells starts early during crypt cell differentiation, being detectable along the whole crypt-villus axis [20, 53]. In conclusion LS174T provides a good *in vitro* model for mucin expression by intestinal goblet cells.

Caco-2 is a well-differentiated cell line with enterocyte-like characteristics developing a brush border [28, 29]. The Caco-2 cells could be cultured until at least 25 dpc [30]. In our study MUC1 mRNA is expressed at a level about equal to the level in jejunum (Fig. 1). MUC2 mRNAs are virtually absent, while MUC3 mRNA is expressed at very high levels, about half of the level in jejunum (Fig. 1). This is in agreement with the fact that enterocytes, which are present in high numbers in small intestine, typically express MUC3 mRNA at high levels, but not MUC2 mRNA which is specifically expressed by goblet cells [20]. As is the case in human jejunum and colon, MUC6 mRNA expression was virtually absent in Caco-2. MUC4 and MUC5A/C mRNAs are expressed in Caco-2 at high levels relative to the small intestine and these are the only two mucins of which the expression does not mimic the expression level in jejunum. However, MUC4 mRNA expression is found in colon, and therefore MUC4 expression in Caco-2 might be reminiscent of the colonic origin of Caco-2. The expression level of MUC5A/C mRNA is much higher in Caco-2 than in jejunum and colon. In other studies it was shown that cell populations in cell lines are often not homogeneous with respect to *in vitro* differentiation potential: HT29 parental cell line has a mix of undifferentiated and differentiated cell-types [54]. Probably, in Caco-2 cells there is a small population of cells with another *in vitro* differentiation potential than towards enterocytes, and thus produces other mucins than those typical for enterocytes.

In Caco-2 the level of MUC1 mRNA was fairly constant, independent of cytodifferentiation (Fig. 1). This seems in contrast to the finding that MUC1 mRNA expression is turned off when enterocytes differentiate from the crypt to the villus tip [25]. In this respect Caco-2 is not a good model for MUC1 expression during differentiation. In studies of the HT29 cell line, selected by methotrexate or 5-fluorouracil treatment, mucins were expressed in an differentiation dependent fashion. Notably MUC1 mRNA and protein were detectable in preconfluence and throughout culture which confirms our findings [55]. In our study, MUC3 mRNA expression shows a peak at 7 dpc and this correlates with the appearance of fully enterocyte-like differentiated Caco-2 cells, and with the formation of the brush border [29–31].

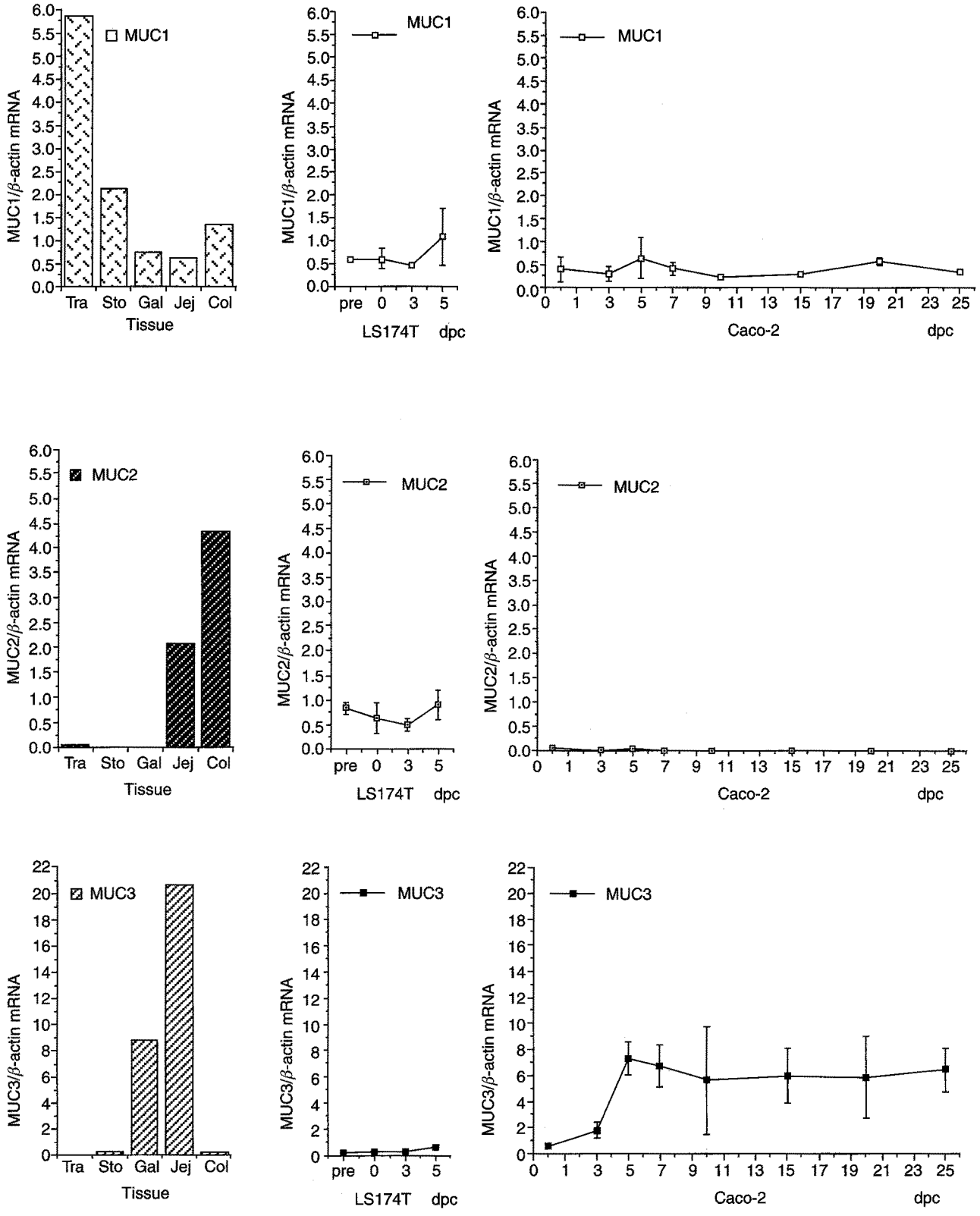

Figure 1. MUC1–6 mRNA levels relative to β-actin mRNA level in Caco-2 cells, in LS174T cells and in tissue. RNA was isolated at preconfluence, 0, 3 and 5 dpc (LS174T) and at 0, 3, 5, 7, 10, 15, 20 and 25 dpc (Caco-2) and from human primary tracheal cell culture (tra), stomach (sto), gallbladder (gal), small (jej) and large (col) intestine. From cell lines, RNA was isolated from two series of experiments. Mucin MUC1–6 mRNA signals were quantified using the PhosphorImager and expressed relative to the signal elicited by β-actin mRNA. Please notice that the scale of the vertical axes of the graphs differ between the different mucin mRNAs.

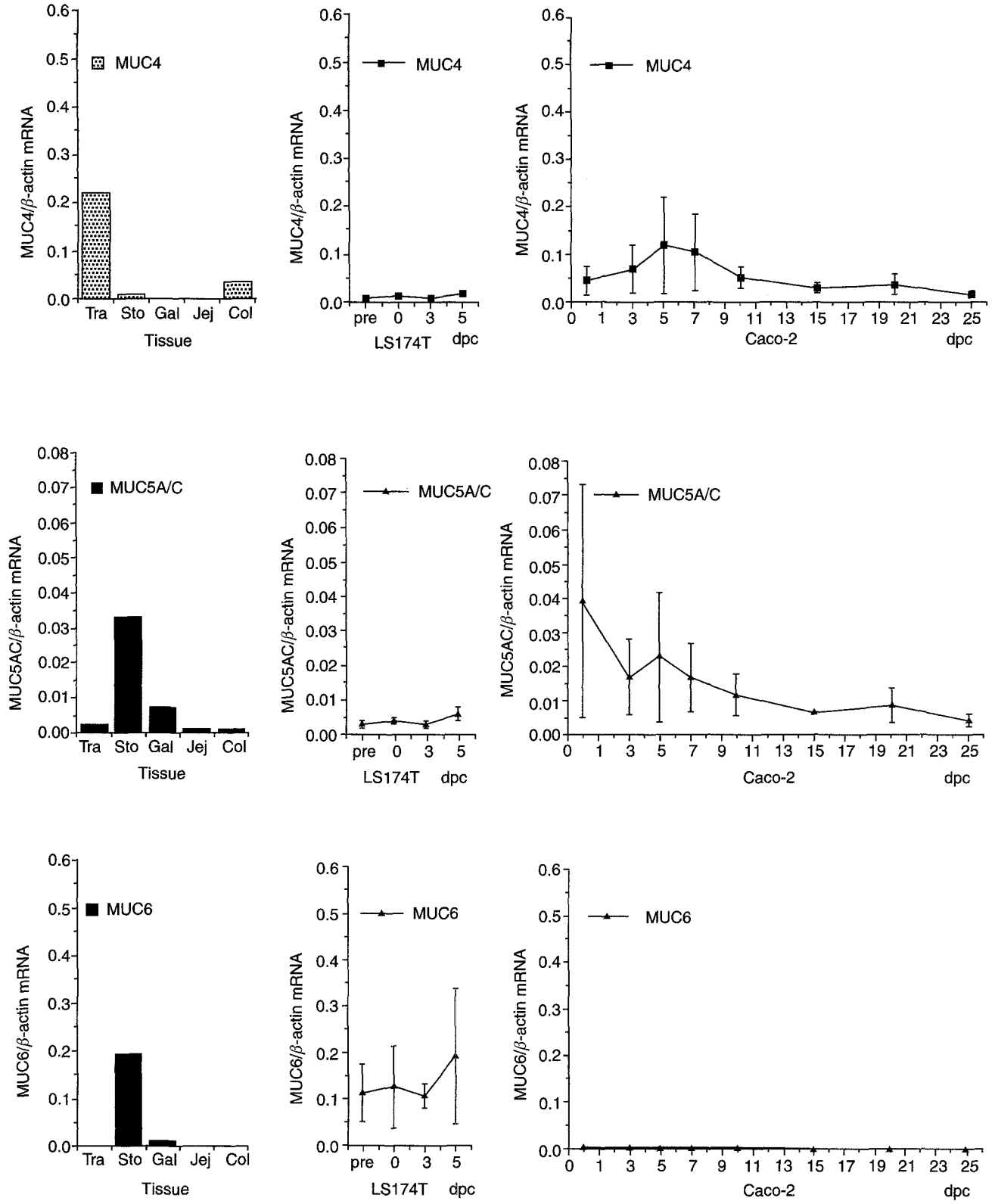

Figure 1. (*continued*)

In the small intestine MUC3 mRNA is highly expressed in enterocytes when they have passed the crypt-villus junction and moved up towards the villus tip [20], also indicating a relationship with enterocyte associated differentiation. Because of the enterocyte-specific expression of MUC3 [20] and its co-expression with brush border glycoproteins like lactase and sucrase-isomaltase [29–31], we suggest that it might be a brush border associated mucin. The complete cDNA sequence of MUC3 is not yet determined and a potential transmembrane sequence may be present. Cell biological experiments should reveal whether MUC3 is brush border associated or secreted. In conclusion Caco-2 provides a good *in vitro* for MUC3 expression and localization in enterocytes of the intestine.

Mucin protein biosynthesis in LS174T and Caco-2 cells

To study mucin precursor biosynthesis, LS174T and Caco-2 cells were metabolically labelled with [^{35}S]methionine/cysteine and [^{3}H]threonine, respectively. Additionally, precursor maturation and secretion of mucins were studied in LS174T cells by labelling with [^{35}S]methionine/cysteine or [^{35}S]sulfate in pulse-chase analyses. Mucins were identified by immunoprecipitations using specific anti-mucin antibodies and analyses on SDS-PAGE.

LS174T and Caco-2 cell cultures were labelled around confluency and at 7 dpc coinciding respectively with highest mRNA expression (Fig. 1). Since LS174T has characteristics of goblet cells containing mucus granules [26, 27], we tested whether it expresses mucin polypeptides specific for goblet cells, namely MUC2, MUC5A/C and MUC6 and also human gallbladder mucin that is expressed in mucous cells of the gallbladder [24]. For comparison we tested whether LS174T cells synthesized MUC3, which is produced predominantly by enterocytes.

After a 30 min pulse-labelling experiment of LS174T cells with [^{35}S]methionine/cysteine, all immunoprecipitated samples were reduced and analysed on the same SDS-PAGE gel to be able to compare apparent molecular masses of the different mucin precursors (Fig. 2, left panel). MUC6 precursor was immunoprecipitated, having a molecular mass of about 400 kDa. This agrees well with the size of the mRNA, which was estimated to be larger than 9.5 kb [19]. MUC3 precursor protein was not detectable in LS174T (Fig. 2, left panel), which agrees with the very low MUC3 mRNA levels in LS174T cells (Fig. 1). MUC5A/C precursors were detected as a double band of approximately 470/500 kDa, in contrast to the single band of about 500 kDa which was immunoprecipitated in similar experiments from gastric explants by Klomp and co-workers [41]. This double band most likely does not reflect degradation as it was seen repeatedly in independent experiments using several protease inhibitors. Because these two bands are always equal in intensity, it is more likely that the double bands

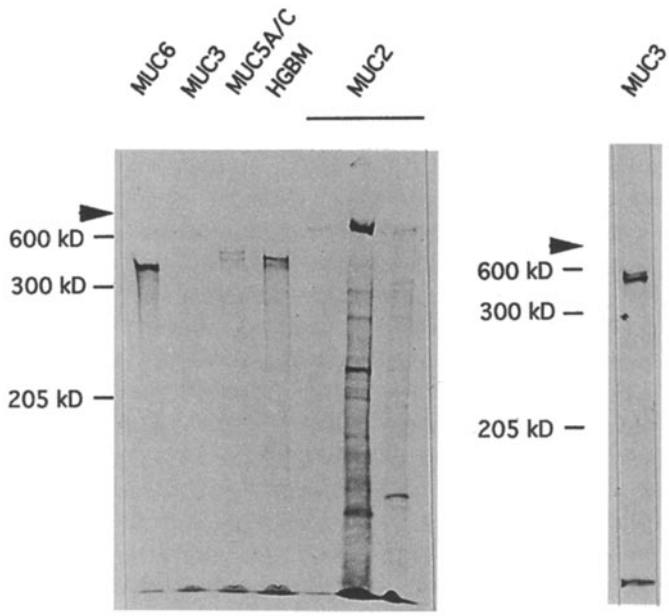

Figure 2. The biosynthesis of mucin precursor proteins in LS174T and Caco-2 cells. LS174T was pulse-labelled for 30 min with [^{35}S]methionine/cysteine (left panel). Mucins were immunoprecipitated from the homogenate using different MUC2 antibodies: monoclonal WE9 and polyclonal anti-RCM and anti-HCM antisera. Precursors of MUC6, MUC3, MUC5A/C and HGBM were immunoprecipitated using respectively anti-M6.1, anti-M3P, anti-RGM and anti-HGBM. Caco-2 was pulse-labelled for 45 min with [^{3}H]threonine (right panel) and MUC3 was immunoprecipitated from the homogenate using anti-MUC3 (anti-M3P). Samples were reduced, analysed by SDS-PAGE and fluorographed to identify the molecular masses of precursors. For LS174T all samples were analysed on one gel. The arrowhead indicates the border between the 3% stacking and the 4% running gel. Molecular mass markers are indicated on the left, i.e. unreduced rat gastric mucin precursor (600 and 300 kDa) and prestained high-molecular mass marker (205 kDa).

reflect allelic variation, showing two precursors of slightly different molecular mass. Allelic variation is very common among mucin genes [5]. Immunoprecipitated MUC2 precursor from LS174T cell homogenates was detected at about 600 kDa on SDS-PAGE using either polyclonal or monoclonal MUC2 antibodies (Fig. 2, left panel). This is similar to the estimated molecular mass of the MUC2 precursor in human small and large intestine [21, 56, 57]. A variety of other faster migrating bands were visible in immunoprecipitations with polyclonal anti-MUC2 antisera, which are not immunoprecipitated by the monoclonal anti-MUC2 antibody. Similar bands were also visible after immunoprecipitation with corresponding pre-immune serum indicating that these bands are aspecific (data not shown). HGBM precursor was detected at about 470 kDa and its mobility was clearly distinguishable from the other mucin precursors, except from the lower band of MUC5A/C. In

LS174T cells, the HGBM precursor has a similar mobility as the HGBM precursor in the human gallbladder and intestine [24, 58]. Thus, in LS174T cells MUC2, MUC5A/C and MUC6 mRNA and proteins were shown to be expressed, and MUC3 mRNA was expressed at low levels whereas MUC3 protein was not detected.

After pulse-labelling for 45 min of Caco-2 cell cultures with [^{3}H]threonine, SDS-PAGE analysis showed that MUC3 precursor was detectable in Caco-2 cell homogenates as a double band at about 550 kDa (Fig. 2, right panel). This double band likely represents allelic variation, since intensities of both bands were similar. MUC3 precursor has a similar molecular mass in Caco-2 cells as in human small intestine, also showing a double band [57]. The prominent expression of MUC3 at protein as well as mRNA level and the differentiation dependent expression in Caco-2 cells therefore implies that Caco-2 may very well function as an *in vitro* differentiation model of MUC3 in enterocytes.

To verify the precursor status of mucins as shown in Fig. 2 (left panel) digestion with endo H was performed. This enzyme cleaves off high mannose N-glycans, which are added early in biosynthesis prior to addition of O-glycans [6]. Each band was demonstrated to be endo H-sensitive (Fig. 3), as indicated by slight mobility shifts. This confirms that all immunoprecipitated bands represent most likely mucin precursor proteins. Importantly, after endo H-treatment HGBM migrates at a position in the SDS-PAGE gel that is clearly distinguishable from all other endo H-treated precursors produced by the LS174T cells shown in Fig. 2. The gene encoding HGBM is not yet identified, but based on the mobility on SDS-PAGE of mucin precursors, HGBM is not identical to MUC2, MUC3, MUC5A/C, or MUC6. The molecular masses of MUC1 and MUC7 precursor protein on SDS-PAGE are about 160–310 kDa and 39 kDa, respectively [12, 59] and therefore very different from the observed apparent molecular mass of HGBM, thus HGBM is not identical to MUC1 or MUC7 either. Alternatively, HGBM could be MUC4, however based on its low RNA expression level in LS174T cells this is not very likely. MUC8 is also an unlikely candidate for HGBM, since MUC8 mRNA is not expressed in the small intestine [17], whereas HGBM precursor is [23]. Therefore, HGBM could be encoded by a yet unidentified human mucin gene, although at present we cannot rule out the possibility that it might be encoded by the *MUC5B* gene. Currently we are in the process of identifying the HGBM gene.

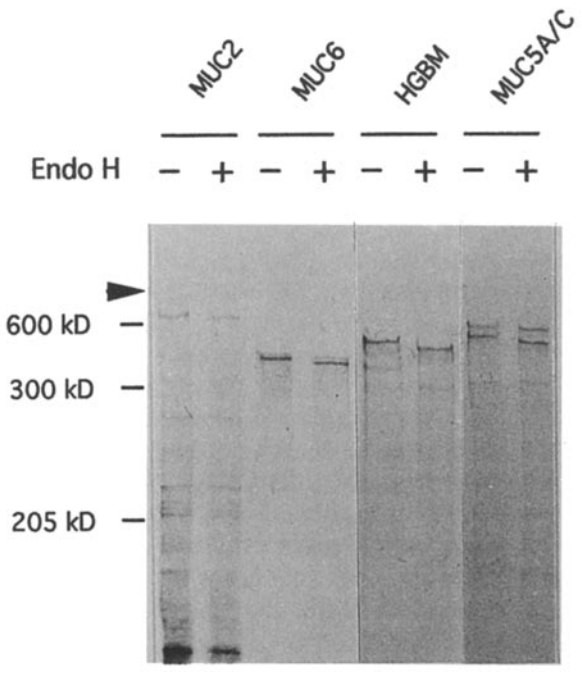

Figure 3. Endo H digestion of mucin precursors. From LS174T, MUC2, MUC6, HGBM and MUC5A/C precursors were immunoprecipitated and analysed on SDS-PAGE as for Fig. 2. The mucin precursors were either incubated with (+) or without (–) endo H prior to analyses on gel.

Thus, metabolic labelling studies demonstrated precursors of MUC2, MUC6, HGBM and MUC5A/C in LS174T and of MUC3 in Caco-2. All precursors can be distinguished by their respective molecular masses as analysed on SDS-PAGE. In both cell lines molecular masses of mucin precursors are comparable to those found in tissue, except for MUC5A/C which shows a double band, suggesting allelic variation.

Metabolic pulse-chase labelling studies were performed to demonstrate relationships between mucin precursor and mature mucins and to show whether they are secretory. In a pulse-chase analysis performed with [^{35}S]methionine/cysteine the MUC2 precursor protein was detected after a short pulse (Fig. 4). After a chase of 20 h the precursor is partly converted into mature MUC2, acquires a mobility in the gel just below the MUC2 precursor band, and is subsequently also secreted into the medium. The identification of mature MUC2 was confirmed by [^{35}S]sulfate labelling of MUC2 (Fig. 4), showing that [^{35}S]sulfate-labelled mature MUC2 has a similar mobility as [^{35}S]methionine/cysteine labelled mature MUC2 and was also secreted. In LS180, the parental cell line of LS174T, the biosynthesis was shown to proceed very similarly: MUC2 precursor and mature mucin, which was secreted, displayed very similar apparent molecular masses on SDS-PAGE of about 600 kDa [60]. Moreover, the biosynthesis of MUC2 appeared very similar to the biosynthesis of MUC2 in human biopsies, as we have shown earlier [21].

The protein precursor of HGBM was detectable at 470 kDa after a short pulse-labelling, but also after the chase-incubation of 4 h together with a mature product with apparent molecular mass of about 600 kDa (Fig. 4).

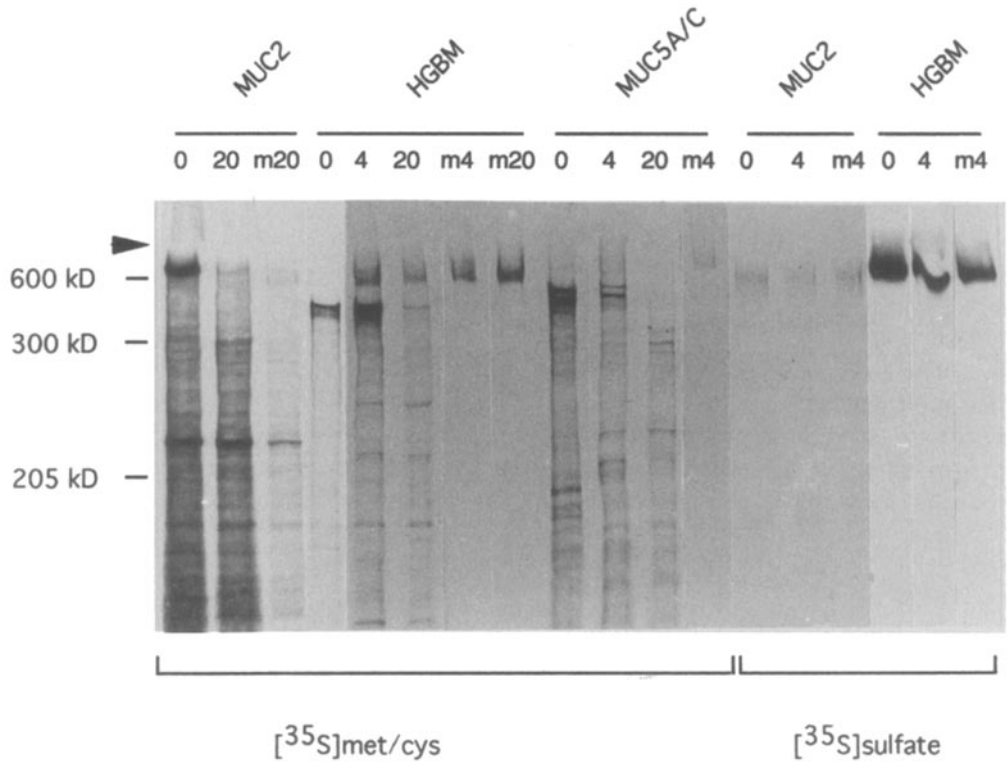

Figure 4. The biosynthesis and secretion of mature MUC2, HGBM and MUC5A/C in LS174T cells. LS174T cells were pulse-labelled with [^{35}S]methionine/cysteine and chase-incubated with complete unlabelled medium for 4 and 20 h. Cells were homogenized after 0, 4 or 20 h chase; medium was collected after 4 or 20 h chase as indicated by m4 and m20. Alternatively, LS174T was pulse-labelled with [^{35}S]sulfate. Cells were homogenized immediately after the pulse or after 4 h chase period, and medium was collected at 4 h chase (m4). Mucins were immunoprecipitated from cell homogenates or culture media using anti-MUC2 (anti-HCM), anti-HGBM and anti-MUC5A/C (anti-RGM) antisera and analysed on reducing SDS-PAGE as for Fig. 2. The chase periods (h) are indicated.

After a 20 h chase-incubation little precursors could be detected, indicated by a faint band at 470 kDa, and mostly mature HGBM was immunoprecipitated from the cell homogenate. In the medium mature HGBM was present, indicating that HGBM was secreted similar as for the human gallbladder [24]. In a pulse-chase study using [^{35}S]sulfate, mature HGBM was labelled and detectable after a pulse of 1 h as a band of about 600 kDa (Fig. 4). After a chase-incubation of 4 h this band was immunoprecipitated from the cell homogenate, but also from the medium, confirming that HGBM is a secretory mucin.

The precursor protein of MUC5A/C was visible after a pulse-incubation with [^{35}S]methionine/cysteine and was also immunoprecipitated after a 4 h chase-incubation from the cell homogenate together with a mature MUC5A/C product of about 600 kDa. This mature MUC5A/C was also secreted as it was immunoprecipitated from the medium. After a chase-incubation of 20 h very little mature MUC5A/C could be detected in the cells and none in the medium (data not shown), indicating that processing and turnover of MUC5A/C was probably more rapid, than was the case for MUC2 and HGBM. Pulse-labelling with [^{35}S]sulfate showed that mature MUC5A/C could be detected as a band at about 600 kDa (data not shown). Our study confirms that MUC5A/C is a secretory mucin as was shown previously by studies of human stomach biopsies [41, 61]. Analysis of mature MUC6 and MUC3, by means of pulse-chase studies, is hampered because all available antibodies were raised against the tandemly repeated peptide sequences, which become masked upon O-glycosylation. Therefore, pulse-chase experiments showing the maturation of MUC3 and MUC6 can only be carried out when antibodies against the respective unique termini of MUC3 and MUC6 are available. For this reason we are presently unable to elucidate the secretory status of these two mucins. However, mature MUC2, HGBM and MUC5A/C were detectable in LS174T cells and were shown to be secreted into the medium displaying a mobility on SDS-PAGE of $\geqslant$ 600 kDa.

The differences between the mobilities of mature mucins and their precursors on SDS-PAGE is surprisingly small. Mucins are generally known to be heavily O-glycosylated and sulfated, which greatly enhances their molecular mass [1, 6]. The small difference in mobility

between precursor and mature mucin was also observed for rat and human MUC2 [21, 39]. Most likely the mobilities of mature MUC5A/C, MUC2 and HGBM is anomalous, when compared with other proteins, due to the high carbohydrate content and to the high negative charge of the mucins, caused by high amounts of sialic acid and sulfate as discussed elsewhere in detail [21, 37, 39, 60, 62]. Since the mobilities of mature mucins are anomalous, no conclusions can be drawn on the actual molecular masses of mature MUC2, MUC5A/C and HGBM as expressed in LS174T compared to human colon.

In conclusion, the LS174T cell line expresses MUC2, which is typically expressed at high levels in the large intestine. The mucins identified in LS174T are expressed early in differentiation and therefore typical for goblet cells, i.e. MUC2, MUC5A/C, MUC6, and HGBM. The metabolic labelling studies are in line with the mRNA data, meaning whenever there is mRNA, the protein is synthesized. LS174T has provided us with an excellent model to demonstrate that HGBM most likely is not one of the mucins cloned so far, and may represent a novel mucin. The enterocyte-like cell line Caco-2 expresses MUC3 at high levels, which is typical for small intestinal enterocytes. MUC3 is expressed relatively late in *in vitro* differentiation of Caco-2 cells, as is the case in the human small intestine [20]. Both cell lines therefore provide excellent *in vitro* models to study the regulation of cell-type specific intestinal mucin expression in response to the cytokines found and drugs used in inflammatory bowel disease.

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