Synthesis and secretion of mucin by the human colonic tumour cell line LS180

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Pulse-chase labelling experiments were performed using the mucin-producing colonic carcinoma cell line LS180. Cells were pulsed with [³H]threonine or [³H]glucosamine and chased in complete media without radiolabel for various lengths of time. From cell and media extracts obtained at each time point, mucin proteins were immunoprecipitated with specific anti-mucin antibodies and analysed by SDS/PAGE and fluorography. At short labelling times with [³H]threonine, without chase, a monomeric thiol-reduction-resistant mucin precursor of apparent molecular mass > 670 kDa was identified. The precursor, in contrast to oligomeric species, was not labelled by [³H]glucosamine but exhibited binding to *Vicia villosa* isolectin B4, suggesting the presence of some core GalNAc residues. Treatment with tunicamycin to inhibit N-glycosylation had no effect on the apparent mass of the precursor. Identity of the mucin antigen with MUC2

mucin was established by immunoprecipitation with antibodies specific for a MUC2 tandem repeat and C-terminal regions. With increasing chase time the precursor was replaced by thiol-reduction-sensitive mucin oligomers that reached peak intracellular radiolabelling with [³H]threonine by 2 h of chase, and then declined. Only oligomeric mucin was secreted into the medium. Secretion of [³H]threonine-labelled mucin was detectable after 2 h of chase and increased as the cytoplasmic mucin label declined. Monensin inhibited [³H]glucosamine incorporation, sialylation and baseline (non-regulated) mucin secretion without affecting initial [³H]threonine incorporation or oligomerization. Oligomerization and Golgi transport are therefore essential early steps in MUC2 mucin secretion. Oligomerization may follow some core O-glycosylation with GalNAc, but precedes elongation of oligosaccharide chains.

INTRODUCTION

Intestinal mucins are large negatively charged glycoproteins which are synthesized, stored and secreted by highly polarized exocrine cells called goblet cells. These cells are recognized by their apical accumulation of mucin storage granules and are also known to produce more than one type of mucin molecule (Neutra and Forstner, 1987; Specian and Oliver, 1991). Once secreted into the intestinal lumen, mucin molecules form a gel that serves as a protective barrier for the underlying mucosal epithelia. This mucus barrier is maintained by slow continual mucin secretion arising from periodic exocytosis of the contents of a single mucin granule (Neutra et al., 1977; Specian and Neutra, 1984; Specian and Oliver, 1991). Regulatory mechanisms of accelerated mucin exocytosis also exist along with this baseline secretion. Goblet cells can rapidly secrete most or all of their stored intracellular mucin in response to a variety of stimuli, including cholinergic stimulation (Specian and Neutra, 1980, 1982; Phillips et al., 1984) and physical irritation (Neutra et al., 1982).

Secretory, gel-forming mucin molecules are disulphide-bonded oligomers and contain up to 80% (w/w) oligosaccharides which are O-linked to threonine and serine residues on the polypeptide backbone (Neutra and Forstner, 1987). At the molecular level, gel formation depends mainly on the formation of hydrophilic non-covalent bonds between the oligosaccharides of oligomeric mucin molecules (Allen, 1981; Carlstedt and Sheehan, 1984; Neutra and Forstner, 1987; Strous and Dekker, 1992). The production of gastrointestinal mucins is a very complex process, and only relatively recently have some detailed studies been

reported. Dekker et al. (1989) and Dekker and Strous (1990), for example, have examined the sequential steps between protein synthesis in the rough endoplasmic reticulum and the final maturation in the Golgi complex of rat gastric mucin. The presence of N-linked oligosaccharides appeared to be necessary for efficient formation of the quaternary structure of the molecule. Also, the major part of the O-glycosylation process took place after oligomerization by inter-chain disulphide bonding of monomeric precursors, each having a molecular mass of about 300 kDa. In contrast, studies of human gastric mucin have suggested that it is synthesized from a 900 kDa monomeric precursor (Dekker et al., 1991). Partial cDNA sequences derived from two human intestinal mucin genes, *MUC2* and *MUC3*, have been cloned (Gum et al., 1989, 1990, 1992). These genes contain tandemly repeated sequences that encode peptides which are rich in threonine (*MUC2*) and threonine and serine (*MUC3*). The repetitive arrays of amino acids are thought to be heavily O-glycosylated and to make up a major portion of the overall structure of the large polymorphic mucus glycoproteins (Toribara et al., 1991; Gum et al., 1992). Both the *MUC2* and the *MUC3* genes are expressed in human colon and small intestine, in colonic tumours and in the LS174T colonic tumour cell line.

To study the sequence of events involved in intestinal mucin synthesis and secretion, we have performed pulse-chase labelling experiments followed by immunoprecipitation with specific anti-mucin antibodies or lectin binding using the mucin-producing human colonic carcinoma LS180 cell line. This cell line was derived from the same adenocarcinoma as the trypsinized variant LS174T (Tom et al., 1976). We show that cultures of LS180 cells make a thiol-reduction-resistant MUC2 mucin precursor of

Abbreviations used: SI mucin, human small intestinal mucin; MRP, threonine- and proline-rich amino acid sequence of the first tandem repeat of MUC2 mucin; MEM, minimum essential medium; endo H, endo- β -N-acetylglucosaminidase; PMSF, phenylmethanesulphonyl fluoride; NP40, Nonidet P-40.

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apparent molecular mass > 670 kDa and a higher-molecular-mass oligomeric mucin, with only the latter being secreted. Inhibition of processing in the medial and trans-Golgi, using the ionophore monensin (Tartakoff, 1983), resulted in an increased cellular level of poorly sialylated, oligomeric mucin and greatly diminished secretion, without affecting initial labelling with [³H]threonine or monomer-oligomer transition.

MATERIALS AND METHODS

Chemicals

L-[3-³H]Threonine, D-[6-³H]glucosamine hydrochloride and D-[1-³H]galactose were from Amersham Corp. (Oakville, Ontario, Canada). EN³HANCE was from DuPont-New England Nuclear (Mississauga, Ontario, Canada). Protein A-agarose, (peanut agglutinin lectin)-agarose, (*Vicia villosa* isolectin B4)-agarose, Nonidet P-40 (NP40), phenylmethanesulphonyl fluoride (PMSF), tunicamycin and endo- β -N-acetylglucosaminidase (endo H) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monensin and laminin (rat L2 yolk sac tumour) were from Calbiochem (La Jolla, CA, U.S.A.). Neuraminidase (protease-free) from *Vibrio cholerae*, N-glycosidase F, O-glycosidase, pepstatin and leupeptin were from Boehringer Mannheim (Laval, Quebec, Canada). Thyroglobulin was obtained from Pharmacia LKB (Uppsala, Sweden).

Human mucin and antibodies

Mucin from a normal human small intestine (SI mucin) was purified according to Mantle et al. (1984a). The antibody to normal human SI mucin was a 40% (NH₄)₂SO₄ fraction of rabbit immune sera and its specificity has been presented in earlier reports (Qureshi et al., 1979; Mantle et al., 1984b; McCool et al., 1990). The polyclonal antibody to MRP (the threonine- and proline-rich amino acid sequence of the first tandem repeat of MUC2 mucin sequenced) (Gum et al., 1989) and the polyclonal antibody to the C-terminal 118 kDa glycopeptide of SI mucin (Mantle and Stewart, 1989) were generously provided by Y. Kim (University of California, San Francisco, CA, U.S.A.) and M. Mantle (University of Calgary, Alberta, Canada) respectively.

Cell culture

The LS180 human colonic carcinoma cell line was obtained from the American Type Culture Collection (ATCC), Rockville, MD, U.S.A. The cells were grown in Eagle's minimum essential medium (MEM) with non-essential amino acids in Earle's balanced salts solution containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) (all from Gibco, Grand Island, NY, U.S.A.). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The cell line was passaged by removing the spent medium, adding fresh medium and dislodging the cells from the surface of the culture flask with a sterile scraper. The cell suspension was pipetted up and down in a sterile pipette to break up clumps, then dispensed into new culture flasks. The subcultivation ratio was 1:4.

Metabolic labelling

LS180 cells were seeded into 6-well (35 mm) culture dishes (Linbro; ICN Biomedicals Inc., Costa Mesa, CA, U.S.A.), with each well containing about 2 × 10⁵ cells in 2 ml of culture medium. The cells were given fresh medium on the third and fifth days of

culture and used for experiments on the sixth day, when they were approximately 70% confluent. Spent medium was removed and the cells washed once with Dulbecco's PBS and then incubated for 45 min at 37 °C in 1 ml of Eagle's MEM containing only 10% of the usual concentration of glucose when [³H]glucosamine or [³H]galactose labelling was to be performed, or without threonine (MEM Selectamine Kit; Gibco) if [³H]threonine labelling was desired. After the 45 min starvation period the media were removed and the cells were incubated for 10 or 30 min at 37 °C in 1 ml of MEM with one-tenth of the usual concentration of glucose and containing 200 µCi/ml [³H]glucosamine (40.4 Ci/mmol) or [³H]galactose (5 Ci/mmol), or in threonine-free MEM containing 200 µCi/ml [³H]threonine (19 Ci/mmol). The radioactive media were removed at the end of the pulse period and the cells were quickly washed twice with 1 ml of PBS containing 5 mM unlabelled glucosamine, galactose or threonine as appropriate. The cells were then incubated in complete LS180 culture medium at 37 °C for various lengths of time ranging from 0 to 24 h. At each time point the media were harvested separately and the adherent cells were lysed at 4 °C for 1 h in the dark in 25 mM Tris/HCl buffer, pH 7.4, containing 1% NP40, 5 mM disodium EDTA, 100 mM NaCl, 0.02% sodium azide, 1 mM PMSF, 10 mM iodoacetamide, 10 µg/ml pepstatin and 25 µg/ml leupeptin. The lysates were then clarified by centrifugation at 11000 g for 4 min in an Eppendorf Microfuge and the supernatants were stored at -70 °C. NP40, disodium EDTA, sodium azide, PMSF, iodoacetamide, pepstatin and leupeptin were added to the harvested media to give the concentrations described above for the lysis buffer and the media, and then stored at -70 °C.

Drug addition during labelling experiments

Monensin was added to give a final concentration of 1 µM in both the labelling and chase solutions. Tunicamycin was used at a final concentration of 30 µg/ml in the 45 min starvation medium and both labelling and chase media.

Immunoprecipitation procedures

Mucin was precipitated from aliquots of cell lysate or media (usually 50–100 µl) in 1.5 ml Eppendorf tubes by incubation with the anti-(SI mucin) antibody for 2 h at room temperature followed by the addition of Protein A-agarose (Sigma P2545) and overnight incubation at 4 °C. The Protein A-agarose had previously been washed three times with immunoprecipitation (IP) buffer (20 mM Tris/HCl, pH 8.0, containing 0.1 M NaCl, 1 mM disodium EDTA, 0.5% NP40 and 0.1 mM PMSF) and then resuspended at 1:1 (v/v) in this buffer. Anti-MRP or anti-(118 kDa glycopeptide) antibodies, or preimmune rabbit serum as a control, replaced the anti-(SI mucin) antibody in some experiments. The samples were then centrifuged at 11000 g for 4 min in an Eppendorf Microfuge and the pellets were washed twice with 1 ml of IP buffer and once with 1 ml of IP buffer without NP40. The pellets were then resuspended in 50 µl of SDS/PAGE sample buffer [containing 2% (w/v) SDS with or without 5% (v/v) 2-mercaptoethanol or 20 mM dithiothreitol (Bio-Rad, Richmond, CA, U.S.A.)] and boiled for 3 min.

Electrophoresis and fluorography

The boiled samples were analysed by SDS/PAGE according to Laemmli (1970) using a 3% stacking gel and a 4% running gel (Miniprotean II; Bio-Rad). Prestained high-molecular-mass standards (116.5 kDa, 205 kDa, reduced; Bio-Rad), laminin

(200 kDa, 400 kDa; reduced) and thyroglobulin (669 kDa; non-reduced) were used as molecular-mass markers. After electrophoresis the gels were fixed in 30% methanol, 10% acetic acid and 10% trichloroacetic acid, then treated with EN³HANCE (Dupont) using the procedure described by the manufacturer. The gels were dried and fluorographed using Kodak X-Omat AR X-ray film (exposure time at -70°C ranged from 4 days to 4 weeks). For some experiments the proteins detected on the fluorographs were quantified using a laser densitometer (Ultrosan XL; Pharmacia LKB).

Lectin treatment

Radiolabelled cell extracts (100 μl) were incubated with (*Vicia villosa* isolectin B4)–agarose or (peanut agglutinin lectin)–agarose (each of which had previously been washed three times with PBS) for 2 h at room temp. After centrifugation at 11000 g for 4 min, supernatants were removed and lectin pellets washed twice with PBS. Lectin-bound glycopeptides were eluted overnight at 4°C with 2 vol. of PBS containing the appropriate competing sugar at a concentration of 100 mM.

Enzyme assays

Neuraminidase digestion

Mucin antigens were immunoprecipitated from radiolabelled cell extracts as described above. After washing with IP buffer (minus NP40) the pellets were suspended in 50 μl of 50 mM sodium acetate buffer, pH 5.5, containing 4 mM CaCl_2 , with or without neuraminidase (10 munits/50 μl). Samples were then incubated for 0, 2 or 4 h at 37°C with shaking, followed by centrifugation to remove supernatants, and the pellets were washed with 1 ml of PBS at room temperature. The pellets were then boiled in 30 μl of SDS/PAGE sample buffer and aliquots of 20 μl were used for SDS/PAGE and fluorography.

Endo H digestion

Mucin antigens were immunoprecipitated from 100 μl portions of [^3H]threonine-labelled cell extracts as described above and boiled for 3 min with 0.1% SDS in 0.2 M sodium acetate buffer, pH 5.5. The supernatants were then diluted until the SDS concentration was 0.04%, and the sodium acetate concentration was adjusted to 0.1 M. An equal volume of 0.1 M sodium acetate buffer, pH 5.5, containing 2 mM PMSF, 4 mM dithiothreitol and BSA (10 $\mu\text{g}/\text{ml}$), with or without endo H (0.5 unit/ml), was added, and the samples were incubated for 18 h at 37°C . The samples were then dialysed against distilled water at 4°C and lyophilized.

N-Glycosidase F digestion

Mucin antigens were immunoprecipitated from 200 μl portions of [^3H]threonine-labelled cell extracts and boiled for 3 min with 1% SDS in 0.2 M sodium phosphate buffer (pH 7.2). The samples were then incubated for 24 h at 37°C in 20 mM sodium phosphate buffer, pH 7.2, containing 10 mM EDTA, 0.5% NP40, 0.1% SDS, 1% (v/v) 2-mercaptoethanol, 1 mM PMSF and 4 units/ml N-glycosidase F.

O-Glycosidase digestion

This was carried out using the conditions described above for digestion with N-glycosidase F, except that O-glycosidase (25 munits/ml) was used as enzyme. Neuraminidase (20 munits/ml) was also present to remove sialic acid residues.

RESULTS

Pulse–chase analysis of mucin synthesis and secretion

LS180 cells were pulsed for 30 min with [^3H]threonine to label peptides and chase-incubated for various lengths of time up to 24 h as described in the Materials and methods section. Radio-labelled proteins from cells and media were immunoprecipitated with anti-(SI mucin) antibody or a control immunoglobulin fraction from preimmune rabbit serum and analysed by SDS/PAGE under non-reducing and reducing conditions. Using the anti-(SI mucin) antibody, fluorographs revealed that cell extracts electrophoresed under non-reducing conditions contained two strong mucin antigen bands: band A, which was present near the top of the separating gel at early chase times and then disappeared, and a more slowly migrating band, B, which was at the junction of the stacking and separating gels (Figure 1a). Minor bands of greater mobility and a faint band at the top of the stacking gel (position C) were also noted (Figure 1a). When immunoprecipitations were carried out with preimmune serum, no bands in positions A, B or C were seen (Figure 1b), although a number of bands of lower molecular mass were present. The strong band at the bottom of the gel coincided with the dye front

Figure 1 Pulse–chase analysis of LS180 mucin (non-reducing SDS/PAGE)

Fluorograms of SDS/polyacrylamide gels showing mucin immunoprecipitation from cell and media extracts (100 μl) using either anti-(SI mucin) antibody or a control IgG fraction prepared from preimmune serum. Cells were pulsed for 30 min with [^3H]threonine and chased for the times indicated. (a) Cell extracts treated with anti-(SI mucin) antibody; (b) control preimmune IgG fraction. (c) Competitive binding: immunoprecipitation of 100 μl of [^3H]threonine-labelled cell extracts (2 h chase) with anti-(SI mucin) antibody in the presence or absence of unlabelled human SI mucin (200 ng of mucin protein/ μl); lane 1, minus SI mucin; lane 2, plus SI mucin. (d) Media: lanes 2–8, anti-(SI mucin) antibody; lanes 1 and 9, control preimmune serum. The arrowhead indicates the junction of the 3% stacking with the 4% separating gel. Bands A, B and C are described in the Results section.

Figure 2 Pulse-chase analysis of LS180 mucin (reducing SDS/PAGE)

Samples were prepared as described in the legend to Figure 1 and subjected to thiol reduction as described in the Materials and methods section. (a) Lanes 1–6, cell extracts immunoprecipitated with anti-(SI mucin) antibody; lane 7, immunoprecipitation with preimmune serum. (b) Media immunoprecipitated with anti-(SI mucin) antibody. Band D is described in the Results section.

and was present in both immune and preimmune precipitates from cell extracts. To confirm that the anti-(SI mucin) antibody was only recognizing cell mucin(s), immunoprecipitation in the presence of unlabelled SI mucin was performed. The SI mucin competitively reduced the precipitation of labelled protein in positions A, B and C (Figure 1c), demonstrating that only mucin antigens were immunoprecipitated. Analyses of media extracts (Figure 1d) showed that labelled mucin appeared in the medium in positions B and C, but not in position A, after a 2 h chase (lanes 2–8), and that B and C were absent from control immunoprecipitates (lanes 1 and 9). Thus an intracellular mucin band of lower apparent molecular mass (band A) is only present at the earliest labelling times. This band is characteristic of an early precursor since it is replaced by more slowly migrating mucin bands which ultimately appear in secretions and therefore represent mature mucin. The amount of mucin appearing in the medium in positions B and C increased with chase time as the amounts of mucin in these positions decreased in cell extracts (Figure 1a). Densitometric analysis (not shown) revealed that about 60% of the 24 h secretion of mucin into the medium had taken place by 6 h of chase.

Thiol reduction gave rise to a single, somewhat diffuse, mucin band from both cell extracts (Figure 2a) and media (Figure 2b) at the position designated D. This mucin band was not present in control immunoprecipitates when the anti-(SI mucin) antibody was replaced by control preimmune IgG (Figure 2a, lane 7). In agreement with the results shown in Figure 1, the intensity of band D in cell extracts decreased with chase time as it increased in media samples. Thus the mucin antigens seen in positions B and C in Figure 1 give rise to a single mucin monomer on reduction. It is noteworthy, however, that only one band was found in all lanes, even in lane 1 which contained the putative mucin precursor band A (Figure 1a). In fact, bands A and D occupied virtually the same position in the non-reducing (Figure 1a) and reducing (Figure 2a) gels, suggesting that band A was not affected by reduction. Thus band A could represent a monomeric precursor of the disulphide-linked oligomers in bands B and C. Oligomerization of mucins is likely to occur in the endoplasmic reticulum, however, while the bulk of the O-glycosidic chains are added later, in the Golgi (Dekker and Strous, 1990). A pre-oligomeric endoplasmic reticulum precursor ought, therefore, to be of lower molecular mass than a monomer that is derived by reduction of mature mucin because it is less

Figure 3 Pulse-chase analysis after a 10 min pulse of [^3H]threonine

Analyses were performed on labelled cellular mucin immunoprecipitated with anti-(SI mucin) antibody at various chase times, as indicated. Other conditions were as in Figures 1 and 2. (a) Non-reducing SDS/PAGE; (b) reducing SDS/PAGE. Bands A–D are described in the Results section.

glycosylated. We considered two possibilities: (1) that the pulse period of 30 min was inappropriate for labelling a true early precursor, and (2) that labelled monomers derived from mature glycosylated mucin by reduction were migrating in an anomalous manner with respect to mass on SDS/PAGE.

To investigate the first possibility, LS180 cells were pulsed with [^3H]threonine for a shorter time, 10 min, in an effort to focus on newly synthesized antigen. To investigate the second possibility, cells were also pulsed for 10 min, in separate experiments with [^3H]glucosamine or [^3H]galactose, to determine which of the immunoreactive mucin products were glycosylated. After each of these pulse periods, a cold chase was carried out for various lengths of time up to 6 h to look at processing and secretion. In non-reduced gels (Figure 3a) it is apparent that band A was the only band labelled by [^3H]threonine at 0 h of chase, while oligomeric bands in positions B and C were labelled at later chase times. In some experiments intermediate bands appeared between A and B (see Figure 5), but these were also converted to band D on reduction (see Figure 6). In reducing gels (Figure 3b) of the same samples shown in Figure 3(a), only band D was present, as before, and it migrated in a similar position to band A. Thus band A was unaffected by thiol reduction. No bands of lower apparent molecular mass were seen. From a comparison with high-molecular-mass SDS/PAGE standards, including pre-stained high-molecular-mass standards from Bio-Rad, thyroglobulin and laminin, band A has an apparent molecular mass of > 670 kDa, when run on either non-reducing (Figures 1a and 3a) or reducing (Figure 3b) gels.

When the 10 min pulse-[^3H]glucosamine-labelled mucin bands (or galactose-labelled bands; results not shown) from cell extracts were examined (Figure 4a) in non-reducing gels, the oligomeric bands B and C were labelled, but there was no signal in the band A area. Band A was therefore not glycosylated or, at least, less well glycosylated than the oligomeric mucins, suggesting that it represents an early mucin precursor. Interestingly, a [^3H]glucosamine-labelled band was present at position B in the zero-chase lane, indicating that carbohydrate was incorporated into oligomers very rapidly, presumably due to the glycosylation of pre-synthesized peptide that had already traversed the endoplasmic reticulum and entered the Golgi. Under reducing conditions (Figure 4b), [^3H]glucosamine-labelled oligomers were replaced by a single monomeric mucin band (band D), as before. Since the mature mucin monomer was more completely glycosylated than the mucin precursor, band A, but nevertheless migrated in the same position after reduction (Figure 3), these

Figure 4 Pulse-chase analysis after a 10 min pulse of [^3H]glucosamine

Analyses are shown of labelled cellular and secreted (medium) mucin immunoprecipitated with anti-(SI mucin) antibody at various chase times, as indicated. Other conditions were as in Figures 1 and 2. (a) Non-reducing SDS/PAGE; (b) reducing SDS/PAGE.

results suggest that the monomers formed by reduction of oligomeric mucin were migrating more rapidly on SDS/PAGE than is appropriate for their mass.

Immunoprecipitation by antibodies to MUC2

Immunoprecipitation of cell extracts, labelled with [^3H]threonine for 10 min, was performed using the antibody to MRP [the threonine- and proline-rich amino acid sequence of the first tandem repeat of MUC2 mucin (Gum et al., 1989)]. The anti-MRP antibody recognized a mucin antigen which migrated in the same position as band A (Figure 5a, lanes 2 and 4), and did not recognize any protein of lower molecular mass than antigen A.

The antibody to the SI mucin 118 kDa glycopeptide (Mantle and Stewart, 1989) is now known to be specific for a region within the unique amino acid sequence of the C-terminus of MUC2 mucin (Xu et al., 1992; Gum et al., 1992). When this antibody was used to immunoprecipitate mucin from cell extracts labelled for 10 min with [^3H]threonine, once again only antigen in the same position as band A was detected (Figure 5a, lane 5). These observations strongly suggested that mucin antigen A is a MUC2 mucin precursor, since a band of the same molecular mass was present after immunoprecipitation by antibodies to both a sequence in the tandem repeat region and the C-terminus of the MUC2 mucin.

To obtain additional evidence that the anti-(SI mucin) antibody recognized MUC2 mucin, 0 and 1 h post-pulse cell extracts (10 min of [^3H]threonine labelling) were immunoprecipitated initially with the anti-MRP antibody; the remaining supernatant was re-immunoprecipitated with the anti-(SI mucin) antibody. Separate, single, immunoprecipitations were also performed with the two antibodies for comparative purposes. Figure 5(b) shows results for the 1 h post-chase sample. Both the anti-MRP and the

Figure 5 Anti-(SI mucin) antibody recognizes MUC2 mucin

LS180 cells were labelled with [^3H]threonine for 10 min and then chased for 0 min lanes 1–6 and 1 h lanes 7–9. Aliquots (100 μl) of cell extracts were immunoprecipitated with control preimmune IgG and the pellets were discarded. Supernatants were then immunoprecipitated with antibodies as indicated, followed by SDS/PAGE and fluorography. (a) 0 min chase; lanes 1 and 2, non-reducing SDS/PAGE; lanes 3–6, reducing SDS/PAGE. (b) 1 h chase; non-reducing SDS/PAGE. Lane 9, anti-(SI mucin) antibody was added following removal of antigen by precipitation with anti-MRP antibody.

anti-(SI mucin) antibodies recognized mucin antigens in positions A and B when used separately (Figure 5b, lanes 7 and 8). In sequential precipitations almost all of the labelled mucin was removed by the first immunoprecipitation with the anti-MRP antibody, and no additional antigens uniquely recognized by the anti-(SI mucin) antibody became apparent (Figure 5b, lane 9). Results at 0 h (not shown) were similar, except that only band A was present.

N- and O-glycosylation

The MUC2 peptide contains a number of potential N-glycosylation sites in addition to O-glycosylation sites (Gum et al., 1992). Digestion of band A with endo H, N-glycosidase F and O-glycosidase had little effect on its mobility on SDS/PAGE (results not shown), supporting the conclusion that this precursor band is sparsely glycosylated. In addition, treatment with tunicamycin during the starvation, pulse and chase periods, in order to inhibit N-glycosylation, had no effect on the mobility of band A. Tunicamycin also inhibited the incorporation of [^3H]threonine into total cellular protein by 43 % and reduced the labelling of mucin bands. In agreement with Dekker and Strous (1990), the transition from monomer to oligomers was slowed, and secretion was inhibited (results not shown). The possibility of core O-glycosylation limited to GalNAc or GalGalNAc was further tested by treatment of 0 and 2 h chase cell extracts (following a 10 min pulse with [^3H]threonine) with agarose-bound lectin (*Vicia villosa* isolectin B4, highly specific for O-linked GalNAc, or peanut agglutinin lectin, highly specific for O-linked Gal β 1,3GalNAc). Supernatants containing unbound mucin and supernatants containing mucin released from lectin-agarose by elution in the presence of competing sugar were incubated with anti-(SI mucin) antibody and the immunoprecipitates were analysed by SDS/PAGE and fluorography. Equivalent aliquots of cell lysate were also immunoprecipitated without subjecting them to lectin binding, to determine the recovery of mucin. About 13 % of the mucin bound to *Vicia villosa* isolectin B4 at 0 min of chase; this decreased to 5 % after 2 h (results not shown). There was less than 5 % binding to the

Figure 6 Effects of monensin

Cells were pulsed for 10 min with [3 H]threonine or [3 H]glucosamine and chased for 30 min, 2 h and 6 h in the presence or absence of monensin (1 μ M). Immunoprecipitations with anti-(SI mucin) antibody of cell and media extracts (100 μ l aliquots for [3 H]threonine; 200 μ l aliquots for [3 H]glucosamine) were performed for each time point and samples were analysed by SDS/PAGE and fluorography. Other conditions were as for Figures 1 and 2. [3 H]Threonine: (a) non-reducing SDS/PAGE, (b) reducing SDS/PAGE; [3 H]glucosamine: (c) non-reducing SDS/PAGE (d) reducing SDS/PAGE.

(peanut agglutinin lectin)-agarose at either time (results not shown). Lack of lectin binding after a 2 h but not a 0 min chase may be due to addition of sialic acid to O-linked GalNAc and O-linked GalNAcGal, thus masking these sugar residues. The binding of 13% of the zero-chase mucin to *Vicia villosa* B4 does suggest, however, that band A contains a small amount of core O-linked GalNAc.

Mucin maturation: effects of monensin

To study the conversion of the putative mucin precursor A into the more mature oligomeric forms of mucin (B and C), LS180 cells were pulsed for 10 min with [3 H]threonine or [3 H]glucosamine and chased in the presence or absence of 1 μ M monensin, which is known to inhibit Golgi processing of many proteins (Tartakoff, 1983). Figure 6 shows that monensin had major effects. The incorporation of threonine into oligomers at 0.5 h

was not affected, but at 2 and 6 h the amount of labelled intracellular mucin was increased (Figure 6a). At the same time, little mucin entered the medium from cells treated with monensin, even after a 6 h chase. Thus monensin greatly reduced the rate of mucin secretion while having comparatively little effect on monomer-oligomer transformation. Under reducing conditions (Figure 6b), mucin from the monensin-treated cells migrated more slowly than that from control cells after 2 and 6 h of chase, but not after 0.5 h. This suggested that impaired glycosylation of [3 H]threonine-labelled mucin occurred in the presence of monensin and that the resulting intracellular mucin had a lower mobility on SDS/PAGE. Furthermore, since mucin was predominantly oligomeric after 0.5 h of chase (Figure 6a), and its behaviour on reducing SDS/PAGE was unchanged by treatment with monensin (Figure 6b), oligomerization must occur before or at an early stage of glycosylation. Results obtained with [3 H]glucosamine (Figures 6c and 6d) supported these conclusions. Monensin greatly reduced incorporation of the carbohydrate label at all time points and slowed the migration rate of intracellular mucin on reducing SDS/PAGE. Oligomers were also labelled by [3 H]glucosamine at 0.5 h under non-reducing conditions (Figure 6c). However, in contrast to its lack of effect on [3 H]threonine-labelled oligomers at this time point, monensin both reduced glucosamine incorporation and caused the remaining label to migrate more slowly in reducing gels (Figure 6d). Therefore the oligomers labelled by [3 H]threonine at 0.5 h were not labelled by [3 H]glucosamine; thus most [3 H]glucosamine incorporation must take place after formation of oligomeric mucin by disulphide bonding.

Neuraminidase treatment

Tumour cell mucins, including LS174T mucin glycoproteins, are defectively glycosylated and often contain an excess of sialic acid residues which contribute significantly to the negative charge of these molecules (Siddiqui et al., 1989) and may cause greater migration during SDS/PAGE (Hilkens and Buijs, 1988). Since the monensin experiments suggested that inhibition of Golgi function could have decreased the negative charge on mucin, experiments were performed to determine whether significant numbers of sialic acid residues were present in the LS180 mucin oligomeric products and if their presence affected mobility on SDS/PAGE. Mucin antigens were initially immunoprecipitated from cells which had been pulsed with [3 H]threonine or [3 H]glucosamine for 10 min and chased for 30 min or 6 h with or without monensin. The samples were then treated with neuraminidase for 2 or 4 h at 37 $^{\circ}$ C, washed with PBS and subjected to reducing SDS/PAGE and fluorography. Figure 7(a) shows that the mobility of the mucin in position D in the 6 h chase [3 H]threonine sample decreased after neuraminidase digestion (lanes 7 and 8) and was in a similar position to the labelled mucin from the monensin-treated sample (lane 6). However, the mobility of the bands in the 30 min chase sample (lanes 1-4) remained unchanged. Incubation in acetate buffer alone had little effect (Figure 7a, lanes 1 and 10). With [3 H]glucosamine as the labelled precursor (Figure 7b), the amount of label in the mucin bands decreased after neuraminidase treatment, showing that a significant amount of the [3 H]glucosamine was incorporated into mucin sialic acid residues. Again, the mucin from the neuraminidase-treated sample chased for 6 h migrated more slowly and in a similar fashion to the mucin from the monensin-treated samples (Figure 7b, lanes 5 and 6). Thus mature LS180 mucin contains sialic acid residues which affect its electrophoretic mobility on SDS/PAGE. In addition, the [3 H]glucosamine-labelled mucin from the earliest

Figure 7 Effects of neuraminidase on mucin immunoprecipitates from control and monensin-treated cells

Mucin antigens were immunoprecipitated from 50 μ l [3 H]threonine-labelled or 100 μ l [3 H]glucosamine-labelled cell extracts (30 min and 6 h chase) with the anti-(SI mucin) antibody, washed, neuraminidase-treated in acetate buffer and analysed by SDS/PAGE and fluorography (see the Materials and methods section). Other conditions were as in Figure 3. (a) [3 H]Threonine. Lanes 1–4, 30 min chase: lane 1, control incubated in acetate buffer for 4 h; lane 2, monensin-treated; lane 3, control incubated in acetate buffer with neuraminidase for 4 h; lane 4, control. Lanes 7–10, 6 h chase: lane 6, monensin-treated; lanes 7 and 8, control incubated in acetate buffer with neuraminidase for 2 and 4 h respectively; lane 9, control; lane 10, control incubated in acetate buffer for 4 h. (b) [3 H]Glucosamine. Lanes 1–3, 30 min chase: lane 1, control; lane 2, control incubated with neuraminidase for 4 h; lane 3, monensin-treated. Lanes 4–6, 6 h chase: lane 4, control; lane 5, control incubated with neuraminidase for 4 h; lane 6, monensin-treated.

chase sample (Figure 7b) was affected by neuraminidase, indicating that it was sialylated. In contrast, the corresponding [3 H]threonine-labelled sample was unaffected (Figure 7a). The mucin labelled by [3 H]glucosamine was clearly more mature than the mucin labelled by [3 H]threonine at this time and, as noted previously, this is probably accounted for by the labelling of mucin apopeptides that were in the medial or trans-Golgi at the time of the pulse.

DISCUSSION

We have used pulse-chase analysis of mucin production in the LS180 cell line to show that the mucin peptide is synthesized as a thiol-reduction-resistant, lightly glycosylated, mucin precursor (band A) with an apparent molecular mass of > 670 kDa on SDS/PAGE. It is highly likely that the mucin antigen identified by our studies is MUC2, since the precursor and more mature mucin oligomers were recognized by antibodies that are specific for a tandem repeat and the C-terminal region of MUC2. Molecular mass estimates of MUC2 apomucin determined by SDS/PAGE after immunoprecipitation with specific antibodies have been reported as 580 kDa (approx.) for the LS174T cell line (Devine et al., 1991), and > 460 kDa and about 400 kDa for a high-mucin-producing variant of it (Ohara et al., 1991). Gum et al. (1992) have sequenced most of human colonic MUC2 mucin cDNA and suggest that the deduced protein has a molecular mass of > 470 kDa. Nevertheless, our estimate of > 670 kDa for

the poorly glycosylated mucin precursor is greater than one might expect from available data. The size discrepancy is not due to disulphide-dependent oligomerization, since we found no evidence of a smaller molecule under stringent reducing conditions. Conformational factors, particularly the adoption of an extended structure, could possibly account for some of the discrepancy by slowing movement through polyacrylamide gels. Tandem repeat regions of a number of mucin peptides, particularly MUC2, are very rich in proline residues (Strous and Dekker, 1992), which favour an extended peptide chain. Reduced SDS binding due to glycosylation could be a factor, but the earliest mucin precursor in the endoplasmic reticulum is likely to be sparsely glycosylated. If early glycosylation were a significant factor one would normally expect that loss of N-glycosylated oligosaccharides would increase mobility on SDS/PAGE, yet mobility was unchanged by treatment with tunicamycin and glycosidases. The addition of core GalNAc residues also seems to be very important in stiffening a linear extended conformation in mucin molecules (Shogren et al., 1989). The timing and location of the initiation of O-glycosylation is not well defined. Studies on the synthesis of rat gastric mucin suggest that initial O-glycosylation is a continuous process, starting on nascent polypeptides in the rough endoplasmic reticulum, but with the major site of core GalNAc addition in a late endoplasmic reticulum compartment or in the cis-Golgi (Dekker and Strous, 1990). There is evidence that some core O-glycosyltransferase activity may be shifted from a Golgi to an endoplasmic reticulum location in colon cancer cells (Egea et al., 1993). In our experiments we were able to show that the monomeric mucin precursor bound *Vicia villosa* isolectin B4 to a small extent, suggesting that some core GalNAc residues were present. No binding to peanut lectin was observed, implying that additional Gal residues had not been added to produce Gal β 1,3GalNAc. Our results therefore suggest that some core O-glycosylation occurs before oligomerization, i.e. very shortly after translation of the peptide. Perhaps early O-glycosylation of some serine and threonine residues could help to stabilize an extended conformation in the centre of the nascent mucin chains so that the cysteine-rich N- and C-terminal domains are kept well separated. Dekker and Strous (1990) reported that oligomerization of gastric mucin precedes O-glycosylation, but also found that oligomerization was not required for initial GalNAc addition. Only 5% of the gastric mucin precursor was retained by a GalNAc-specific lectin column.

Disulphide-dependent oligomerization appears to be an essential prerequisite for secretion in LS180 cells, since we found no evidence that mucin monomers entered the medium at any time. Similar findings have been reported for mucins from the stomach (Dekker and Strous, 1990). Oligomerization primarily takes place in the endoplasmic reticulum, aided frequently by association with molecular chaperones (Gething and Sambrook, 1992). When we treated cells with monensin to disrupt Golgi activity, [3 H]threonine incorporation into the mucin precursor and oligomerization were not affected as one might expect for pre-Golgi events. In addition, mucin secretion was inhibited, while [3 H]threonine-labelled oligomeric mucin accumulated intracellularly. Taken together, these findings suggest that transport of oligomeric mucin through the Golgi complex was greatly reduced.

In accordance with its expected site of action, monensin greatly reduced glycosylation of oligomeric mucin, as reflected in reduced [3 H]glucosamine incorporation and slowed migration in reducing SDS/PAGE. We were able to show that the change in migration rate was due to the absence of sialic acid by producing an identical effect with neuraminidase. Similar effects

of monensin on mucin synthesis and secretion have been reported by others. In rat gastric cells, monensin inhibited the glycosylation and sulphation of mucin, and secretion was also greatly reduced (Dekker and Strous, 1990). Sialylation of the epithelial mucin MAM6 in the ZR-75-1 human breast cancer line was also inhibited by monensin (Hilkens and Buijs, 1988). With the breast cancer cell line MCF-7, mucin intermediates having molecular masses between those of the precursor and the mature mucin were present after monensin treatment (Linsley et al., 1988). Also, treatment of rat mammary 13762 adenocarcinoma cells with monensin reduced the conversion of immature into mature ascites sialoglycoprotein-1 (ASGP₁), a cell surface sialomucin, by disrupting the cellular compartment involved in β 1-4 galactosylation of ASGP₁. Sulphation and the transport of the mucin to the cell surface were reduced (Spielman et al., 1988; Hull and Carraway, 1989). The role of sialic acid in promoting an anomalously rapid migration rate on SDS/PAGE was not initially appreciated in our studies and caused us to have difficulty in distinguishing between precursor and reduced monomeric forms of mature mucin. Subsequent experiments showed clearly, however, that the mucin precursor was not labelled, as were mature oligomers, by [³H]glucosamine, and its electrophoretic behaviour was not affected by monensin or neuraminidase. Anomalous migration of glycoproteins on SDS/PAGE due to sialic acid has been reported previously for two mucins: MUC1 from the human breast cancer cell line ZR-75-1 (Hilkens and Buijs, 1988), and a mucin from the HT29-LMM human colon cancer cell line (Dohi et al., 1993).

Our studies show that a [³H]threonine-labelled precursor is present in the cells after a 10 min pulse, and that monensin-insensitive oligomers are present at 0.5 h of chase. [³H]Threonine-labelled mature MUC2 mucin was first detected in the culture medium after a 2 h chase. These parameters suggest that folding, oligomerization and transport through the endoplasmic reticulum occur within the first 30 min after incorporation of amino acid label, whereas passage through Golgi compartments, the budding off of condensing granules and the movement of secretory granules to the cell apex takes a minimum of 1.5 h in LS180 cells. Mucin secretion continued for several hours, with about 60 % of the total 24 h secretion taking place in the first 4 h. Autoradiographic studies in unstimulated rabbit intestinal goblet cells suggested that intracellular transport from the endoplasmic reticulum to the apical surface may take 3 h (Oliver and Specian, 1991) rather than the 1.5 h indicated by our experiments. Intracellular transport also seems to be slower in normal gastric mucous cells (Dekker and Strous, 1990), since [³H]methionine-labelled mucin did not appear in the medium before 4 h of chase (Dekker et al., 1989). Further experiments would be required, however, to determine whether these differences are dependent on the type of cell and/or site of origin.

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