Regulated and unregulated pathways for MUC2 mucin secretion in human colonic LS180 adenocarcinoma cells are distinct

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We have shown previously [McCool, Forstner and Forstner (1994) *Biochem. J.* **302**, 111–118] using pulse–chase labelling of mucin with [³H]threonine that LS180 colonic tumour cells synthesize and secrete MUC2 without the addition of secretagogues. Treatment of the LS180 cells with monensin to disrupt Golgi function was also found to inhibit baseline secretion almost completely. In this paper we show that addition of nocodazole to inhibit microtubule assembly reduced baseline secretion by 53 % over a 6 h chase period. In contrast, cytochalasin D did not affect the rate of unstimulated mucin synthesis or secretion, suggesting that baseline secretion is not influenced by disruption of actin microfilaments. In addition, regulated mucin secretion by LS180 cells was studied in response to carbachol, phorbol 12-myristate 13-acetate and A23187. Mucin released in response to secretagogues behaved identically on SDS/PAGE to that secreted under baseline conditions. T84 cells

and the B6 subclone of the HT29 cell line responded in a similar manner to LS180 cells and secreted high-molecular-mass mucin which included MUC2 and behaved like LS180 mucin on SDS/PAGE. Neither monensin nor nocodazole significantly affected secretagogue-stimulated mucin secretion. Since these compounds inhibited secretion of labelled mucin under baseline conditions, mucin released by secretagogues must have come from a separate, unlabelled mucin pool in stored granules. Cytochalasin D, on the other hand, caused the release of small amounts of stored mucin, suggesting that actin microfilaments participate in regulated exocytosis. Thus two kinds of mucin secretion occur in LS180 cells. Unregulated secretion depends upon continuous transport of mucin granules from Golgi vesicles to the cell surface and does not utilize stored mucin, whereas regulated secretion involves the release of mucin from storage granules and is not affected by microtubule or Golgi disruption.

INTRODUCTION

Mucins, the major component of the mucous gel that lines and protects the surface of the intestinal epithelia, are produced in polarized exocrine cells called goblet cells. These cells store mucin in apically located granules and are able to synthesize more than one type of mucin molecule [1]. The gel-forming mucin molecules are large negatively charged glycoproteins composed of monomeric components linked together by inter-chain disulphide bonds. They contain up to 80 % (w/w) oligosaccharides which are largely O-linked to threonine and serine residues on the polypeptide core. cDNA sequences derived from the cloning of several human intestinal mucin genes, namely *MUC2* [2,3], *MUC3* [4] and *MUC6* [5], indicate that these threonine and serine residues are located in regions containing multiple tandem repeats in the coding sequence. Cysteine residues which are involved in disulphide bonding are mainly located in the poorly glycosylated N- and C-terminal regions. The protein backbones of mucins are synthesized in the rough endoplasmic reticulum, where post-translational modifications such as folding and the initiation of oligomerization also take place. Most O-glycosylation and extension of oligosaccharide chains occurs in the Golgi region, with the addition of terminal sialic acid residues, fucose and sulphate taking place in the trans-Golgi [6,7]. Newly synthesized mucin molecules are sorted into condensing vacuoles which undergo transition to mature storage granules during transport to the apical surface of the cell. Eventually granule membranes fuse with those of the plasma membrane and granule contents are released into the intestinal lumen by exocytosis.

From studies of the movement of labelled granules in intestinal goblet cells, mucin secretion is thought to take place in two ways. Baseline secretion is accomplished by the periodic exocytosis of single mucin granules that tend to follow a peripheral path from the Golgi region to the apical cell surface [1,8]. Treatment with nocodazole to disrupt microtubule structure [1] impeded this process in rabbit colonic explants, implying that microtubules are necessary for baseline mucin granule movement. In addition, actin microfilaments at the apical surface appeared to act as a barrier to exocytosis, since granular movement to the apical surface was accelerated after treatment with cytochalasin D [9]. Regulatory mechanisms of accelerated mucin secretion also exist, and the number of secretagogues capable of stimulating mucin secretion is quite large [6]. In general, intestinal goblet cells rapidly secrete some or all of their mucin contents, including that stored in centrally located granules, in response to cholinergic stimulation [10–12]. More recently, mucin-secreting cell lines derived from human colonic adenocarcinomas have been used as models for studies of mucin exocytosis. It has been shown that T84 cells [13] and the N2, Cl.16E and B6 subclones of the HT29 cell line [14–17] release mucin in response to cholinergic stimulation. Protein kinase C also seems to be involved in regulating mucin secretion, since phorbol 12-myristate 13-acetate (PMA) stimulated secretion from T84 cells [13] and from the HT29-N2 subclone [14,18]. Furthermore, when the calcium ionophore A23187, which raises intracellular Ca²⁺ levels, was used in conjunction with PMA even greater secretion took place [13].

Recently we have used another colonic carcinoma cell line, LS180, to study the biosynthesis and baseline secretion of MUC2

Abbreviations used: SI mucin, human small intestinal mucin; MRP, the threonine- and proline-rich amino acid sequence of the first repeat of MUC2 mucin; MEM, minimum essential medium; PBS, Dulbecco's phosphate-buffered saline; NP40, Nonidet P-40; PMA, phorbol 12-myristate 13-acetate.

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mucin [19]. Using pulse-chase labelling with [^3H]threonine and [^3H]glucosamine we have shown that MUC2 mucin is synthesized as a monomer of apparent molecular mass $> 600\,000$ Da in the rough endoplasmic reticulum and that only oligomeric mucin is secreted. Also, in the presence of monensin, which affects Golgi transport and processing [20], incompletely processed mucin accumulated in the cells and secretion was greatly inhibited. The mucin was shown to be deficient in sialic acid residues and as a consequence migrated more slowly than completely processed mucin on reducing SDS/PAGE.

We now show by experiments involving the immunoprecipitation of labelled mucin with specific anti-mucin antibody that LS180 cells are capable of regulated as well as baseline secretion and that the mucin secreted in response to stimulation by carbachol, PMA and A23187 behaved identically on SDS/PAGE to that secreted constitutively. Using monensin, nocodazole and cytochalasin D we show, however, that there are significant differences in the roles played by Golgi vesicles, microtubules and microfilaments in baseline and regulated mucin secretion.

MATERIALS AND METHODS

Chemicals

L-[^3H]Threonine and D-[^3H]glucosamine hydrochloride were from Amersham Corp. (Oakville, Ontario Canada). EN 3 HANCE was from Dupont-New England Nuclear (Mississauga, Ontario, Canada). Protein A-agarose, Nonidet P-40 (NP40), PMSF, the calcium ionophore A23187 (free acid), PMA, carbachol, colchicine and cytochalasin D were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monensin was from Calbiochem (La Jolla, CA, U.S.A.), nocodazole was from Aldrich Chemical Co. Inc. (Milwaukee, WI, U.S.A.) and pepstatin and leupeptin were from Boehringer Mannheim (Laval, Quebec, Canada).

Human mucin and antibodies

Mucin from a normal human small intestine (SI mucin) was purified as described previously [21]. The antibody to normal human SI mucin was a 40% $(\text{NH}_4)_2\text{SO}_4$ fraction of rabbit immune sera and its specificity has been presented in an earlier report [13]. The antibody to MRP, the threonine- and proline-rich amino acid sequence of the first repeat of MUC2 mucin [22], was generously provided by Y. Kim (University of California, San Francisco, CA, U.S.A.).

Cell culture

The LS180 and T84 cell lines were obtained from the American Type Culture Collection (A.T.C.C.), Rockville, MD, U.S.A. The B6 subclone of the HT29 human colonic tumour cell line [17] was obtained from Dr. K.-M. Kreusel (Institut für Klinische Physiologie, Klinikum Steglitz der Freien Universität Berlin, D-1000 Berlin, Germany). LS180 cells were grown in Eagle's minimum essential medium (MEM) with non-essential amino acids in Earle's balanced salts solution containing 10% (v/v) fetal bovine serum; T84 cells in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium with 4.5 g of D-glucose/l and Ham's F12 nutrient mixture containing 5% (v/v) fetal bovine serum; and HT29-B6 cells in RPMI 1640 minus glucose but containing galactose (4.5 g/l) and 10% (v/v) fetal bovine serum. The various culture media also contained penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$); all cell culture reagents were from Gibco, Grand Island, NY, U.S.A. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The LS180 cell line was passaged by removing the spent medium, adding fresh

medium and dislodging the cells with a sterile scraper. The cell suspension was pipetted up and down in a sterile pipette to break up clumps, and then dispensed into new flasks. T84 and HT29-B6 cell lines were passaged by trypsinization of the adherent cells with 0.05% trypsin/0.53 mM EDTA, pH 7.4 (Gibco).

Metabolic labelling

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 2×10^5 cells in 2 ml of culture medium. LS180 and T84 cells were given fresh medium on the third and fifth days of culture and used for experiments on the sixth day, when they were approx. 70% confluent. Cultures of HT29-B6 cells were used after they had been confluent for 1 week. Spent medium was removed and the cells were washed once with Dulbecco's PBS, and then incubated for 45 min at 37 °C in 1 ml of Eagle's MEM containing 10% of the usual concentration of glucose when [^3H]glucosamine labelling was to be performed, or without threonine (MEM Selectamine Kit; Gibco) before [^3H]threonine labelling. After the starvation period the media were removed and the cells were incubated for 10 or 30 min at 37 °C in 1 ml of MEM containing only 10% of the usual concentration of glucose and 200 $\mu\text{Ci}/\text{ml}$ [^3H]glucosamine (40.4 Ci/mmol), or in threonine-free MEM containing 200 $\mu\text{Ci}/\text{ml}$ [^3H]threonine (15–19 Ci/mmol). The radioactive media were then removed and the cells quickly washed twice with 1 ml of PBS containing 5 mM unlabelled glucosamine 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 6 h. At each time point the media were harvested separately and the adherent cells lysed at 4 °C for 1 h 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 $\mu\text{g}/\text{ml}$ pepstatin and 25 $\mu\text{g}/\text{ml}$ leupeptin. The lysates were then clarified by centrifugation at 11000 *g* for 4 min in an Eppendorf Microfuge and 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 were then stored at –70 °C.

In other experiments LS180 cells were continuously labelled for 72 h with [^3H]glucosamine (20 $\mu\text{Ci}/\text{ml}$), and T84 and HT29-B6 cells for 48 h with [^3H]glucosamine (30 $\mu\text{Ci}/\text{ml}$), prior to mucin secretion assays and immunoprecipitation.

Drug addition during pulse-chase labelling experiments

Monensin, nocodazole and cytochalasin D were added to give final concentrations of 1 μM , 10 μM and 10 μM respectively in labelling and/or chase solutions as described for each experiment.

Immunoprecipitation procedures

Mucin molecules were precipitated from aliquots of cell lysate or media (usually 50–100 μl) in 1.5 ml Eppendorf tubes using the anti-(SI mucin) antibody or the anti-MRP 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), then resuspended 1:1 (v/v) in this buffer. 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 μ l of SDS/PAGE sample buffer containing 2% (w/v) SDS with or without 5% (v/v) 2-mercaptoethanol and boiled for 3 min.

Electrophoresis and fluorography

The boiled samples were subsequently analysed by SDS/PAGE [23] using a 3% stacking gel and a 4% running gel (Miniprotein II; Bio-Rad). Prestained high-molecular-mass standards (116.5 kDa, 205 kDa) (Bio-Rad) and rat L2 yolk sac laminin (200 kDa, 400 kDa) (Calbiochem) were used as molecular mass markers on gels run under reducing conditions, and thyroglobulin (669 kDa) (Pharmacia LKB, Uppsala, Sweden) was used on gels run under non-reducing conditions. After electrophoresis the gels were fixed in 30% (v/v) methanol, 10% (v/v) acetic acid and 10% (v/v) trichloroacetic acid, then treated with EN³HANCE (Dupont) using the procedure described by the manufacturer. The gels were then dried and fluorographed using Kodak X-Omat AR X-ray film (exposure time at -70°C usually 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).

Mucin secretion assays

Secretion assays were carried out as described previously for T84 cells [13]. For LS180 cells, MEM with non-essential amino acids in Earle's balanced salts solution was used as the secretion medium; for the HT29-B6 cells, after 1 week of confluence, RPMI 1640 was used. Reagents added to cell cultures were dissolved directly in the appropriate medium except for PMA, A23187, nocodazole, cytochalasin D, colchicine and monensin, which were initially dissolved in DMSO and then diluted in culture medium. The small amount of DMSO (0.1% at most) present in the media had no effect on control cell cultures. Immunoassay of mucin released into the secretion medium was carried out according to [13].

Histology

LS180 cells were grown on 30 mm diameter Millicell-HA filters (0.45 μ m pore) (Millipore, Bedford, MA, U.S.A.) using the culture medium described earlier. Attached cells were washed twice with PBS and then fixed for 1 h at room temperature in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The cells were then washed twice with phosphate buffer and post-fixed in aqueous 1% osmium tetroxide for 1 h at room temperature. This was followed by a rapid rinse in distilled water, dehydration through graded acetones to absolute acetone, and embedding in Epon/araldite. Sections (1 μ m) were cut using a Reichert Ultracut E and then stained using 1% Toluidine Blue in 1% sodium borate. Ultra-thin sections (80 nm) were also cut from selected blocks, collected on copper grids and stained with uranyl acetate and Sato's lead. Sections were photographed using a Philips 400T electron microscope (Philips, Eindhoven, The Netherlands).

RESULTS

Morphology of LS180 cells in culture

Examination of thin (1 μ m) sections of mucin-producing LS180 cell cultures prepared after fixation and Toluidine Blue staining showed that 20–25% of the cells in individual sections were goblet-like in appearance, with many storage granules and a nucleus located to one side of the cell. Electron micrographs of

two cells are shown in Figures 1(a) and 1(b). The cells did not have a classical goblet-like shape with an expanded granule zone at the apical pole. This could be because LS180 cells bear a closer resemblance to mucin-producing cells from the lower crypt region of the colon than to surface cells [24], but other factors, such as shaping by neighbouring cells, may also be important.

Effects of nocodazole and cytochalasin D on baseline secretion

Figure 2 illustrates an experiment in which LS180 cells were labelled with [³H]threonine for 10 min and chased for up to 6 h, and cell extract and media proteins specifically immunoprecipitated by an antibody to intestinal mucin. When analysed by SDS/PAGE under non-reducing conditions, a mucin band (A) was seen at the earliest time period; this was subsequently replaced at later time periods by a more slowly migrating band (B) and mucin at the top of the stacking gel (C). These results are identical to those shown previously [19], where we determined that band A is a non-reducible monomeric precursor of MUC2 and that B and C represent disulphide-linked oligomers of band A. A duplicate set of culture wells of cells were treated during the pulse and chase periods with nocodazole at a concentration (10 μ M) which is known to disrupt microtubule activity in rabbit colonic goblet cells [25]. Fluorographs show that mucin was synthesized as usual in the presence of nocodazole, with a band at position A at early chase times and mucin at positions B and C present at later times (Figure 2a). As shown previously [19], only oligomeric mucin was secreted into the culture medium (Figure 2b). Secretion into the culture medium was somewhat diminished at 2 h (Figure 2b), and from densitometric analysis total mucin secretion after a 6 h chase was inhibited 53% by nocodazole treatment. A higher concentration of nocodazole (0.1 mM) was found to be toxic. These results are consistent with autoradiographic evidence [25] showing that microtubules play a role in the movement of newly synthesized mucin towards the apical cell surface. Together with our previous finding that monensin virtually abolished baseline secretion of [³H]threonine-labelled mucin [19], they are also consistent with the concept that unregulated, basal, secretion depends on a continuous flow of mucin granules from the trans-Golgi zone.

To investigate the role of microfilaments in baseline mucin production, cells were pulsed for 10 min with [³H]threonine and chased for various times up to 6 h with or without 10 μ M cytochalasin D, an inhibitor of microfilament structural organization. This was followed by immunoprecipitation with the anti-(SI mucin) antibody and analysis by SDS/PAGE and fluorography. Results showed no significant effect of cytochalasin D on mucin synthesis and secretion (not shown). The experiment was then repeated, increasing the [³H]threonine pulse to 30 min and having cytochalasin D present during the chase period only. These experimental conditions are similar to those used [9] to demonstrate an increase in the rate of mucin granule movement to the apical surface in the presence of cytochalasin D. Results (Figure 3) again showed no significant change in the rate of mucin synthesis or secretion from LS180 cells in the presence of cytochalasin D.

Stimulated (regulated) mucin secretion

To determine if LS180 cells were capable of releasing their mucin contents as a result of specific secretagogue stimulation, cells were incubated for 30 min with PMA (10 μ M) and A23187 (10 μ M) (see the Materials and methods section). Figures 1(c) and 1(d) show that treatment resulted in a large decrease in the number of granules containing mucin and in the appearance of large, empty vesicles which presumably result from fusion of

Figure 1 Electron micrographs of LS180 goblet-like cells

(a, b) Sections of LS180 goblet-like cells in culture, unstimulated; (c, d), cells after treatment for 30 min at 37 °C with PMA (10 μM) and A23187 (10 μM). Bar = 1 μm (magnification × 4500).

granule membranes during compound exocytosis and the release of granule mucin at the apex of the cells. We noted, however, that not all granules were exocytosed and that some cells were more responsive to stimulation than others. With carbachol (1 mM), a generally less potent secretagogue [13], there was little evidence of granule loss, although some granules at the apical surfaces of cells had partially emptied their contents (results not shown). Stimulation of exocytosis in HT29-N2 goblet cells is associated with the appearance of similar large vesicles in the apical cytoplasm [15]. Estimation by immunoassay of the amounts of mucin secreted into the culture media by the LS180 cells prior to fixation for electron microscopy showed a 6.1-fold stimulation with A23187 + PMA (control, 445; PMA + A23187, 2704 ng of mucin protein/ml of secretion medium), and a 3.1-fold stimulation with carbachol (1401 ng of mucin protein/ml) (means of three samples of each). The concentrations of A23187 and PMA used (10 μM of each) were lower than those used [18] to empty HT29-N2 goblet cells.

Similar trends were obtained when mucin secretion assays were carried out on cells which had been cultured with [³H]-glucosamine for 72 h in an effort to label centrally located granules before secretagogue addition (Figure 4, lanes 1, 4, 9 and 10). The mucin released in response to PMA and A23187 had the

same electrophoretic mobility on SDS/PAGE as that secreted under baseline conditions (cf. Figure 2b). Using carbachol (1.0 mM) as secretagogue also caused mucin release (Figure 4, lanes 8 and 11), though the level of stimulation was less than that in response to PMA and A23187, as anticipated.

Effects of monensin and nocodazole on secretagogue-stimulated mucin release

The [³H]glucosamine-labelled cells were also used in mucin secretion assays in which the cells were preincubated for 15 min at 37 °C with or without monensin (1 μM), and then incubated for 30 min at 37 °C with PMA and A23187 in the presence or absence of monensin. The mucin released over the 30 min period was immunoprecipitated and analysed by SDS/PAGE and fluorography (Figure 4). Monensin treatment for 45 min did not inhibit mucin release in response to PMA and A23187. This suggests that mucin released upon stimulation was that which was already mature and probably stored in granules prior to drug addition, since the same concentration of monensin greatly inhibited the processing of newly synthesized mucin and its secretion (Figure 6, [19]). This experiment was repeated with unlabelled cells using, in addition, carbachol (1 mM) as a

Figure 2 Effect of nocodazole on baseline mucin secretion

Cells were pulsed for 10 min with [3 H]threonine and chased for the times indicated in the presence or absence of nocodazole (10 μ M). Analyses were performed for each time point on labelled cellular mucin (from 50 μ l aliquots) and secreted mucin (from 100 μ l aliquots) immunoprecipitated with anti-(SI mucin) antibody. Non-reducing SDS/PAGE of (a) cell extracts: lanes 1–4, without nocodazole; lanes 5–8, with nocodazole; and (b) media extracts: lanes 1, 3 and 5, with nocodazole; lanes 2, 4 and 6, without nocodazole. Gels of cell extracts (see Figure 2a) were exposed to film in the same cassette for the same length of time. The arrowhead indicates the junction of the 3% stacking gel with the 4% separating gel.

secretagogue and quantifying the amounts of mucin released by immunoassay. Table 1 shows data which are in agreement with the results of Figure 4, indicating that monensin did not interfere with regulated mucin secretion. There was no effect on carbachol-induced secretion and only minor inhibition (11%) with PMA and A23187. Control mucin release in this experiment was increased somewhat in the presence of monensin (25%), but the reason for this is not known.

To confirm that monensin did not affect stimulated mucin release, secretion assays were repeated using cells which had been pulsed for 10 min with [3 H]threonine followed by a 2 h 'cold' chase. At this point labelled mucin should be nearing the apical cell surface ([19]. The amounts of labelled mucin released during the 30 min stimulation period were small as detected by SDS/PAGE and fluorography, and monensin had no significant effect (results not shown). Quantification of released mucin by immunoassay showed a 6-fold stimulation with PMA plus

Figure 3 Effect of cytochalasin D on baseline mucin secretion

Cells were pulsed for 30 min with [3 H]threonine, then chased for the times indicated in the presence or absence of cytochalasin D (10 μ M). Analyses were performed for each time point of labelled cellular and secreted mucin (from 100 μ l aliquots) immunoprecipitated with anti-(SI mucin) antibody. Other conditions were as for Figure 2. Non-reducing SDS/PAGE of (a) cell extracts, (b) media. Lanes 1, 3, 5 and 7, without cytochalasin D; lanes 2, 4, 6 and 8, with cytochalasin D.

A23187, and again no inhibition in the presence of monensin. Thus the mucin that was being secreted upon stimulation was already mature and stored, since the response was not dependent on a continuous supply of mucin from the Golgi.

When nocodazole was used at a concentration of 10 μ M in secretion assays, it also did not affect mucin release over the time period of the experiments (Figure 4; Table 1). Carbachol-induced mucin secretion was not affected and secretion in response to PMA and A23187 was decreased by only 10%. This again implies that mucin already stored in granules was released on secretagogue stimulation, since secretion of newly synthesized mucin was inhibited by the same concentration of nocodazole (Figure 2).

Colchicine, which also depolymerizes microtubules, was used to study regulated mucin secretion in T84 cells, a second mucin-secreting adenocarcinoma cell line (Table 2). Colchicine when used at a concentration of 10 μ M has been shown [8] to inhibit baseline mucin secretion, but not cholinergically stimulated secretion, from the goblet cells of rabbit colonic explants. In our experiments we used a higher concentration (0.1 mM) of colchicine under conditions that result in depolymerization of microtubules in T84 cells [26]. There was no difference in the

Figure 4 Monensin and nocodazole do not affect secretagogue-stimulated mucin release

LS180 cells were continuously labelled with [^3H]glucosamine (20 $\mu\text{Ci}/\text{ml}$) for 72 h. Cells were then washed twice with PBS, warmed to 37 $^{\circ}\text{C}$ and incubated for 15 min at 37 $^{\circ}\text{C}$ with or without nocodazole (10 μM) or monensin (1 μM). Media were then replaced by fresh media with or without nocodazole or monensin and containing A23187 (10 μM) plus PMA (10 μM) or carbachol (1 mM), where appropriate, and the cells were incubated for 30 min at 37 $^{\circ}\text{C}$. The media were harvested and 100 μl aliquots were used for immunoprecipitation using the anti-(SI mucin) antibody followed by analysis using SDS/PAGE and fluorography (conditions as for Figure 2). Lanes 1–8, non-reducing; lanes 9–11, reducing SDS/PAGE. Lanes 1, 2, 3, 7 and 9, unstimulated; lanes 4, 5, 6 and 10, A23187 + PMA-stimulated; lanes 8 and 11, carbachol-stimulated. Lanes 2 and 5, nocodazole-treated; lanes 3 and 6, monensin-treated.

amount of mucin released by stimulation with carbachol or PMA + A23187, again indicating that regulated exocytosis does not depend on intact microtubules.

Effect of cytochalasin D on regulated mucin secretion

Immunoassays of mucin released into media collected during the chase period in studies of baseline secretion showed that cytochalasin D caused a 33% increase in mucin secretion even though the amount of labelled mucin was unchanged (Figure 3), suggesting that the increment in released mucin detectable by immunoassay was derived from unlabelled storage granules. When cytochalasin D was tested in mucin secretion assays with or without the addition of mucin secretagogue, the total amounts of mucin released by both control and secretagogue-stimulated LS180 cells were significantly increased (Table 3; $P < 0.01$). The effect on control cultures appeared to be a little greater than that on cultures treated with secretagogues. The action of cytochalasin D occurred rapidly. Even during the 15 min preincubation period, cytochalasin D caused a small but significant ($P < 0.01$) increase in mucin release (control, 496.7 ± 14.1 ; +cytochalasin D, 635.0 ± 32.0 ng of mucin protein/ml; $n = 6$).

Stimulation of mucin secretion by the T84 and HT29-B6 cell lines

To determine whether other human colonic adenocarcinoma cell lines responded to secretagogues in the same way as the LS180

Table 1 Effect of monensin or nocodazole on secretagogue-stimulated mucin release by LS180 cells

Cells were washed twice with PBS warmed to 37 $^{\circ}\text{C}$, then incubated for 15 min at 37 $^{\circ}\text{C}$ in secretion medium with or without nocodazole (10 μM) or monensin (1 μM). Media were then replaced by fresh media with or without nocodazole or monensin and containing secretagogues where appropriate. The cells were then incubated for 30 min at 37 $^{\circ}\text{C}$ and the amounts of mucin released into the media during this period were measured by immunoassay. Mucin release is given by means $\pm$ S.E.M. for six wells of cultured cells in each case. For stimulation results, control = 100%.

Additions	Mucin protein (pg/ μg of cell protein)	Stimulation (%)
None	407.5 $\pm$ 4.8	100
Nocodazole (10 μM)	445.0 $\pm$ 18.9	109
Monensin (1 μM)	511.3 $\pm$ 33.3	125
Carbachol (1 mM)	920.6 $\pm$ 38.6	226
Carbachol (1 mM) + nocodazole (10 μM)	1074.6 $\pm$ 46.5	263
Carbachol (1 mM) + monensin (1 μM)	938.5 $\pm$ 52.0	230
PMA (10 μM) + A23187 (10 μM)	2210.0 $\pm$ 77.7	542
PMA (10 μM) + A23187 (10 μM) + nocodazole (10 μM)	1985.4 $\pm$ 28.7	487
PMA (10 μM) + A23187 (10 μM) + monensin (1 μM)	1963.1 $\pm$ 27.5	482

Table 2 Effect of colchicine on secretagogue-stimulated mucin release by T84 cells

Cells were washed twice with PBS at room temperature and then incubated in culture medium in the presence or absence of colchicine (0.1 mM) for 30 min at 4 $^{\circ}\text{C}$ followed by 2 h at 37 $^{\circ}\text{C}$. Media were then removed and the cells incubated in secretion medium with or without colchicine for 15 min at 37 $^{\circ}\text{C}$. Media were then replaced by fresh secretion media containing colchicine and/or secretagogues where appropriate. The cells were then incubated for 30 min at 37 $^{\circ}\text{C}$ and the amounts of mucin released into the media during this period measured by immunoassay. Mucin release is given by means $\pm$ S.E.M. for six wells of cultured cells in each case. For stimulation results, control = 100%.

Additions	Mucin protein (pg/ μg of cell protein)	Stimulation (%)
None	179.00 $\pm$ 6.71	100
Colchicine (0.1 mM)	193.33 $\pm$ 5.58	108
Carbachol (1 mM)	548.17 $\pm$ 17.06	306
Carbachol (1 mM) + colchicine (0.1 mM)	545.01 $\pm$ 18.54	304
PMA (10 μM) + A23187 (10 μM)	1360.00 $\pm$ 39.34	760
PMA (10 μM) + A23187 (10 μM) + colchicine (0.1 mM)	1431.67 $\pm$ 51.81	800

Table 3 Effect of cytochalasin D on secretagogue-stimulated mucin release by LS180 cells

Conditions were the same as described in the legend to Table 1. Results are means $\pm$ S.E.M. for six wells of cultured cells in each case. For stimulation results of assays done with or without cytochalasin D (10 μ M), control = 100% in each case.

Additions	Mucin protein (pg/ μ g of cell protein)	Stimulation (%)	Increase in total secretion due to cytochalasin D (%)
None	427.6 $\pm$ 17.1	100	—
Carbachol (1 mM)	1137.9 $\pm$ 85.6	283	—
PMA (10 μ M) + A23187 (10 μ M)	2517.2 $\pm$ 97.4	589	—
Cytochalasin D (10 μ M)	585.8 $\pm$ 33.1	100	37
Carbachol (1 mM) + cytochalasin D (10 μ M)	1465.5 $\pm$ 91.5	250	29
PMA (10 μ M) + A23187 (10 μ M) + cytochalasin D (10 μ M)	3172.4 $\pm$ 114.7	540	26

Figure 5 Mucin immunoprecipitated from T84 and HT29-B6 cell cultures after secretagogue stimulation

(a) T84 and (b) HT29-B6 cells were incubated with [3 H]glucosamine (30 μ Ci/ml) for 48 h. Cells were then treated as described for LS180 cells in the legend to Figure 4, and stimulated to secrete mucin with either carbachol (1 mM) or A23187 (10 μ M) plus PMA (10 μ M) for 30 min at 37 $^{\circ}$ C; the media were then harvested. Aliquots (100 μ l from T84; 50 μ l from HT-29-B6) were then immunoprecipitated using the anti-(SI mucin) antibody followed by analysis using SDS/PAGE and fluorography (conditions as for Figure 2). Lanes 1–3, non-reducing; lanes 4–6, reducing SDS/PAGE. Lanes 1 and 4, control; lanes 2 and 5, carbachol-treated; lanes 3 and 6, (PMA + A23187)-treated.

cells, i.e. with the secretion of similar high-molecular-mass oligomeric mucin, cultures of HT29-B6 and T84 cells were labelled for 48 h with [3 H]glucosamine and the labelled media were collected after stimulation with carbachol or PMA + A23187. Analyses after immunoprecipitation with the anti-(SI mucin) antibody (Figure 5) revealed that mucin secreted from

Figure 6 T84 and HT29-B6 cells in culture synthesize MUC2 mucin

T84, HT29-B6 and LS180 cells were pulsed for 30 min with [3 H]glucosamine and chased for 1 h. Aliquots (100 μ l) of cell extracts were immunoprecipitated with control rabbit preimmune serum and the pellets discarded. Supernatants were then immunoprecipitated with either anti-(SI mucin) or anti-MRP antibody followed by reducing SDS/PAGE and fluorography. Lanes 1, 2, 7 and 8, LS180; lanes 3 and 4, T84; lanes 5 and 6, HT29-B6. Lanes 1, 3, 5 and 8, anti-(SI mucin) antibody; lanes 2, 4, 6 and 7, anti-MRP antibody.

these cell lines behaved like that secreted from LS180 cells on non-reducing and reducing SDS/PAGE.

T84 and HT29-B6 cells in culture make MUC2 mucin

Since the LS180 cells produce MUC2 mucin [19], it was of interest to determine if T84 and HT29-B6 cells also express and secrete MUC2 mucin. Figure 6 shows that immunoprecipitation of labelled cell extracts with either the anti-(SI mucin) antibody or the anti-MRP antibody, which recognizes the first tandem repeat region of the MUC2 peptide, gave rise to mucin bands from all three cell lines in the same position (band D) on reducing gels. Therefore T84 and HT29-B6 cells express MUC2. The intensity of the bands from samples immunoprecipitated with the anti-(SI mucin) antibody was greater than with the anti-MRP antibody. This could be due to the fact that anti-MRP does not recognize glycosylated mucin as well as the anti-(SI mucin) antibody. Mucin immunoprecipitated from all three cell lines with the anti-(SI mucin) antibody gave rise to a discrete band (D)

on reducing SDS/PAGE in the same position as that recognized by the anti-MRP antibody. It is interesting to note that mucin immunoprecipitated from normal human colonic explants with an antibody raised against human colonic mucin migrated as a discrete band of similar molecular mass on reducing SDS/PAGE, and that the prominent secretory mucin of the colon appears to be MUC2 [27].

DISCUSSION

Cells generally secrete proteins either through a non-regulated constitutive pathway, in which proteins enter vesicles without concentration at the trans-Golgi and pass directly to the cell surface for immediate exocytosis, or by a regulated pathway, in which proteins are concentrated in granules that are stored and released only upon secretagogue stimulation. Both pathways can exist in the same cell [28]. In the case of the intestinal goblet cell, classical constitutive secretion via a non-storage, vesicular, route contributes little to mucin secretion because for both baseline (unregulated) and stimulated (regulated) secretion, mucin is condensed within granules that appear to be morphologically identical to other storage granules, and share the same storage compartment [1,6]. This is likely also to be true of mucin secretion from the LS180 cell. Our pulse-chase experiments showed that, after a pulse of [3 H]threonine, a period of about 2 h elapsed before labelled mucin could be detected in the culture medium by immunoprecipitation techniques ([19], and Figure 2). This period is more characteristic of condensation and transfer via a granule route, since direct vesicular transfer is a more rapid process [28]. The goblet cell is not unique in releasing secretions from apparent storage granules in a non-regulated manner. Similar findings have been reported in neurotransmitter cells [29] and parotid glandular cells [30]. In both cell types, baseline exocytosis involves granules that are released continually from a reserve storage granule pool. In contrast, our results with monensin and nocodazole indicate that mucins released in baseline and regulated secretions are derived from substantially separate granule pools. We reported previously that treatment with monensin to disrupt Golgi transport and processing abolished baseline release of labelled mucin into the medium [19], and in the present paper we have shown that monensin has no effect on regulated mucin secretion. These findings imply that the mucin secreted in response to stimulation was already mature and stored in granules prior to treatment with monensin, while baseline (unregulated) secretion depends completely on the continued transport of granules from the Golgi to the cell surface. Furthermore nocodazole had little effect on stimulated mucin secretion from the LS180 cells (Figure 4, Table 1) and colchicine had no effect on stimulated mucin secretion from another colonic adenocarcinoma cell line, T84 (Table 2). In contrast, pulse-chase experiments carried out in the presence of nocodazole demonstrated a 53% inhibition of baseline mucin secretion from LS180 cells. Thus microtubules play a significant role in the transport of newly synthesized mucin during baseline secretion but not of mucin secreted in response to secretagogue stimulation. These results are in agreement with autoradiographic evidence [8,25] from colonic explants of rabbits showing that baseline granule movement, but not accelerated movement, was dependent on intact microtubules. Taken together, they indicate that non-regulated secretion depends on the steady release and transport of granules containing newly synthesized and rapidly turned over mucin which is not significantly mixed with mucin from a stored granule pool.

How much of the newly synthesized cell mucin enters stored secretory granules for subsequent release upon stimulation is not

known. However, after continuously labelling LS180 cells with [3 H]glucosamine over a 72 h period, the cells were able to release labelled mucin in response to carbachol, PMA and A23187, indicating that a portion of the regulated storage pool must be replenished on a continuous basis. The mucin secreted in response to secretagogues behaved identically on SDS/PAGE to that secreted under baseline conditions, suggesting that the product that is incorporated into the two pools is likely to be similar, at least for MUC2.

The apical granule mass of the colonic goblet cell is overlaid with a layer of F-actin-containing filamentous cytoplasm which is very thin. Disruption of microfilaments in this network by cytochalasin D treatment caused an acceleration of baseline secretion but not of regulated secretion in rabbit colonic goblet cells [9]. When mucin secretion was measured by immunoassay in our experiments with LS180 cells, addition of cytochalasin D increased mucin release from both unstimulated and stimulated cell cultures. However, when baseline secretion was measured in pulse-chase experiments using [3 H]threonine to label mucin peptide, increased secretion was not seen, implying that cytochalasin D principally accelerated mucin release from an unlabelled storage compartment. The small response seen with the immunoassay measurements, and the fact that this response was not enhanced by secretagogues, nevertheless make it difficult to conclude with confidence that microfilaments impede secretion of stored mucin. Cytochalasin D could also cause an increase in permeability at the junctions between cells, causing washout of presecreted mucin from the cell surface into the culture medium. Indeed, actin has been localized by immunocytochemical studies to the cytoplasm close to cell junctions in rat intestinal goblet cells [31].

Two other colonic tumour cell lines, T84 and HT29-B6, also secreted mucin in response to stimulation by carbachol and PMA + A23187, and the mucin behaved similarly on SDS/PAGE to that from LS180 cells. These two cell lines, like the LS180 cell line, were found to produce mucin which includes MUC2. This observation agrees with findings of Hollingsworth et al. [32], who showed that MUC2 mRNA is expressed in T84 and LS180 cells, and also with those from our own laboratory (D.-H. Hong and G. G. Forstner, unpublished work) showing that the mRNA is also expressed in HT29-B6 cells. These three carcinoma cell lines therefore behave like normal colonic goblet cells, since they produce MUC2 mucin and secrete mucin in response to stimulation by the same secretagogues.

In summary, colonic adenocarcinoma cell lines appear to be useful models for studying the normal regulation of human intestinal mucin secretion.

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