Roles of calreticulin and calnexin during mucin synthesis in LS180 and HT29/A1 human colonic adenocarcinoma cells

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Molecular chaperones are presumed to associate with large secretory mucin glycoproteins during their synthesis in the endoplasmic reticulum (ER), but have not been identified to date. We decided to look for possible involvement of the chaperones calreticulin (CRT) and calnexin (CLN) during synthesis of two similar gastrointestinal mucins, MUC2 and MUC5AC. Pulse–chase labelling of MUC2 and MUC5AC with [^{35}S]methionine/cysteine ([^{35}S]Promix) was performed using LS180 and HT29/A1 colonic carcinoma cell lines and was followed by immunoprecipitation with anti-mucin and anti-chaperone antibodies. The precipitated labelled mucin precursors were analysed by SDS/PAGE and autoradiography. Using antibodies specific for each mucin, newly synthesized monomeric precursors of both MUC2 and MUC5AC were detected after a 15 min pulse and then disappeared as oligomers were formed during a 2 h chase period. Only homo-oligomers of MUC2 and MUC5AC were present in the cells. Using anti-CRT, the MUC2 monomeric precursor and oligomer were co-precipitated from both cell lines after a 15 min pulse and the oligomer less strongly after a 0.5 h chase, but there was little co-precipitation after a 2 h chase. At this time, MUC2 immunoprecipitated by anti-MUC2 was completely oligomerized and was endo-β-*N*-acetylglucosaminidase-resistant, indicating that the mucin had reached the Golgi region. MUC2 co-precipitated with CRT at zero time and 0.5 h was endo-β-*N*-acetylglucosaminidase-sensitive; therefore CRT must have associated with MUC2 in the ER. Treatment with tunicamycin (TUN) diminished the binding of MUC2 to CRT, suggesting a requirement for initial N-glycan addition during this process. Using anti-CLN, only a weak co-precipitation of MUC2, compared with that seen with anti-CRT, was detected in LS180 cells. In contrast with the findings for MUC2, there was no co-precipitation of MUC5AC with CRT or CLN from either cell line at the various time points. In conclusion, CRT and CLN appear to be involved in MUC2 synthesis at the stage of folding and oligomerization in the ER. Since no interaction of the chaperones with MUC5AC was detected at a similar stage of synthesis, these two structurally similar secretory mucins seem to have different chaperone requirements in the ER.

Key words: chaperones, mucin folding and assembly.

INTRODUCTION

The internal surface of the gastrointestinal tract is covered by a protective gel that is rich in secreted mucus glycoproteins (mucins). MUC2, the major mucin secreted by human colonic goblet cells [1], and MUC5AC, which is secreted by the surface mucous cells of the human stomach [2], are both large oligomeric glycoproteins which can be reduced to their monomeric constituents by interchain-disulphide-bond cleavage. They are heavily O-glycosylated, with most oligosaccharide chains being O-linked to threonine and serine residues in the central tandem-repeat regions of the polypeptide core [3]. The N- and C-terminal regions are enriched in cysteine residues, the positions of which are highly conserved between MUC2 and MUC5AC [4,5]. Some of these cysteine residues are involved in stabilizing folding via intrachain-disulphide-bond formation. The terminal regions also contain four cysteine-rich domains, D1, D2 and D3 being N-terminal and D4, C-terminal. They show more than 60% similarity in amino acid sequence between MUC2 and MUC5AC and are also similar to the domains of the human pro-von Willebrand factor [5–7]. As these domains are known to be involved in disulphide-bond formation and multimerization [8,9], it is possible that the D domains of the mucins play a similar role in oligomerization. In agreement with this hypothesis, it has been shown recently [10] that a peptide corresponding to the rat Muc2 C-terminus undergoes disulphide-mediated dimerization. Potential N-glycosylation sites are also located in the N- and C-terminal regions, and some are known to be occupied, since monomeric precursors of both MUC2 and MUC5AC contain N-glycans [1,2]. From radiolabelled-amino-acid pulse–chase studies, using explants of normal human colon [1] and cultures of colonic adenocarcinoma cells [11,12], it is clear that MUC2 is initially synthesized in the endoplasmic reticulum (ER) as a monomeric precursor which migrates with an apparent molecular mass of approx. 600000 Da on SDS/PAGE. Addition of high-mannose N-linked oligosaccharides, folding of the polypeptide and initial oligomerization are also ER-related events, whereas most O-glycosylation, along with extension and completion of oligosaccharide chains, occurs in the Golgi region [11,13]. This is followed by packaging of the mucin into secretory granules, either for storage or for direct secretion at the apical surface of the colonic goblet cell [3,14]. The colonic tumour cell lines LS174T, LS180 and HT29/A1, which are known to produce MUC2 in culture, also synthesize the gastric mucin MUC5AC, which is not significantly expressed in normal colon ([11,12,15]; D. J. McCool, unpublished work). In both gastric explants and the tumour cells, MUC5AC is initially synthesized as an N-glycan-containing monomeric precursor of apparent molecular

Abbreviations used: ER, endoplasmic reticulum; CRT, calreticulin; CLN, calnexin; EMEM, Eagle's minimum essential medium; endo H, endo β-*N*-acetylglucosaminidase; TUN, tunicamycin; CAS, castanospermine; PDI, protein disulphide-isomerase; CHO, Chinese-hamster ovary.

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mass 500000 Da, as determined by SDS/PAGE, and then oligomerizes in the ER by the formation of interchain disulphide bonds ([2,13]; D. J. McCool, unpublished work). Recent findings have also indicated that oligomers formed in LS174T cells by both MUC2 [16] and MUC5AC [17] are probably dimers.

In general, proteins in the ER fold and undergo subunit assembly with the assistance of a number of chaperones such as calreticulin (CRT), calnexin (CLN), the immunoglobulin-binding protein (BiP) and protein disulphide-isomerase (PDI) [18,19]. Chaperones prevent the aggregation of unfolded and misfolded proteins and play a role in quality control of the ER synthetic pathway [20]. CLN and CRT function as chaperones in a similar manner, although CLN is a type 1 membrane protein, whereas CRT is soluble, is present in the ER lumen, and contains a C-terminal KDEL (Lys-Asp-Glu-Leu) ER retrieval signal [21]. However, CRT shares extensive sequence identity with the luminal domain of CLN [21], and both proteins are unique as chaperones, since they preferentially behave as lectins, interacting specifically with partially trimmed monoglucosylated N-linked oligosaccharides on newly synthesized protein molecules [22–24]. They recognize glycan chains of the form $Glc_1Man_9GlcNAc_2$ and bind with similar affinities [25]. It has also been reported [23,25] that, after initial binding occurs via the glycan, CLN probably associates in a more stable fashion by peptide interaction with peptide moieties on the surface of the incompletely folded protein. The protein then remains associated with the chaperone until it has folded correctly and lost the conformational features responsible for the attachment.

It is not known if chaperones are involved during the synthesis of large mucin molecules. We decided to look for possible involvement of CLN and/or CRT during the synthesis of MUC2 and MUC5AC in the ER. Pulse–chase labelling of MUC2 and MUC5AC in the mucin-producing cell lines, LS180 and HT29/A1, followed by immunoprecipitation with antibodies to MUC2, MUC5AC, CLN or CRT, was carried out. In each cell line, homo-oligomers of the two mucins were immunoprecipitated with the anti-mucin antibodies, but no hetero-oligomers were detected. CRT appeared to be involved during the synthesis of MUC2. The chaperone bound to the newly synthesized mucin monomer and oligomer in both cell lines, but once the mucin had completely oligomerized and moved to the Golgi region for further processing, the chaperone was no longer bound. With anti-CLN there was a weak co-precipitation of MUC2 at the same times that co-precipitation with CRT was found. However, there was no detectable binding of either chaperone to MUC5AC at the same stage of synthesis in the ER, suggesting that the two secretory mucin molecules, although structurally similar, have different ER chaperone requirements.

MATERIALS AND METHODS

Chemicals

A cell labelling mix containing L-[^{35}S]methionine and L-[^{35}S]cysteine ([^{35}S]Promix) was from Amersham Corp. (Oakville, Ontario, Canada). TUN, castanospermine (CAS), pepstatin, Protein A–agarose, goat anti-mouse IgG–agarose, iodoacetamide and Eagle's minimum essential medium (EMEM) lacking methionine and cysteine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nonidet P40 was from ICN Biomedicals Inc. (Aurora, OH, U.S.A.). Endo-β-*N*-acetylglucosaminidase (endo H) and protease-inhibitor-cocktail tablets (Complete™ and Complete™ minus EDTA) were from Boehringer Mannheim (Laval, Quebec, Canada). Digitonin and 1-deoxynojirimycin hydrochloride were from Calbiochem (La Jolla, CA, U.S.A.).

Antibodies

A rabbit polyclonal antibody, raised in our laboratory, against normal human small-intestinal mucin has been described previously [11,15]. It recognizes MUC2 monomers and oligomers in both LS180 and HT29/A1 cells. A polyclonal antibody to MRP (the threonine- and proline-rich amino acid sequence of the first tandem repeat of MUC2 mucin) was raised in rabbits according to the procedure described in [26]. A monoclonal antibody to deglycosylated human gastric mucin recognized MUC5AC [27]. Rabbit polyclonal anti-CLN (Catalogue no. SPA-860, lot 806402) was from StressGen Biotechnologies Corp. (Victoria, BC, Canada) and rabbit polyclonal anti-CRT was obtained from Affinity Bioreagents Inc. (Golden, CO, U.S.A).

Cell culture

The LS180 colonic carcinoma cell line was obtained from the American Type Culture Collection, Manassas, VA, U.S.A. The cells were grown in EMEM with non-essential amino acids in Earle's balanced salt solution, and contained 10% (v/v) fetal-bovine serum. HT29/A1, a mucin-producing subclone of the HT29 human colonic tumour cell line, was obtained from Dr. K.-M. Kreusel (Institut für Klinische Physiologie, Universitätsklinikum, Freie Universität Berlin, Berlin, Germany). It has been described previously as HT29/B6 [28]. HT29/A1 cells were cultured in RPMI-1640 medium containing 10% fetal-bovine serum. Culture media also contained penicillin (100 units/ml) and streptomycin (100 μg/ml); all cell-culture reagents were from Life Technologies (Burlington, Ontario, Canada). Cultures were maintained at 37 °C in a humidified atmosphere of air/CO_2 (19:1).

Metabolic labelling

LS180 cells were seeded into six-well (35 mm-diameter) culture dishes (Corning, NY, U.S.A.); each well contained approx. 2×10^5 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 approx. 70% confluent. 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 EMEM lacking methionine and cysteine (starvation medium). The medium was removed and the cells were incubated for 15 min at 37 °C in 1 ml of EMEM without methionine and cysteine, but containing 200 μCi/ml [^{35}S]Promix (1000 Ci/mmol). The radioactive medium was removed at the end of the pulse period and the cells were quickly washed twice with 1 ml of Dulbecco's PBS containing 5 mM unlabelled methionine and cysteine. The cells were then incubated in complete LS180 culture medium at 37 °C for various lengths of time ranging from 0 to 4 h. Labelling of HT29/A1 cells was carried out in a similar manner, except that RPMI-1640 was used instead of EMEM. At each time point the medium was removed and the adherent cells were lysed at 4 °C for 1 h, in the dark, in Dulbecco's PBS, pH 7.4, lacking Ca^{2+} and Mg^{2+}, but containing 10 mM iodoacetamide, one tablet of Complete™ minus EDTA/10 ml, 10 μg/ml pepstatin and either 1% (v/v) digitonin or 1% (v/v) Nonidet P40. The lysates were then clarified by centrifugation at 11000 ***g*** for 4 min in an Eppendorf Microfuge and the supernatants were used for immunoprecipitation. Protease inhibitors were added to the harvested media to give the concentrations described above for the lysis buffers, and the media were then stored at −70 °C.

Immunoprecipitation procedures

Mucin was precipitated from 100 μl aliquots of cell lysates in 1.5 ml Eppendorf tubes; lysates were precleared with rabbit preimmune serum by incubation for 1 h at room temperature and mixed with Protein A–agarose (Sigma P2545) for 30 min. {The Protein A–agarose had been washed previously three times with either immunoprecipitation buffer I [Dulbecco's PBS minus Ca^{2+} and Mg^{2+}, pH 7.4, containing 0.2 % (w/v) digitonin, 1 mM PMSF and 0.02 % (w/v) NaN_3], which was used with cell lysates prepared with buffer containing 1 % digitonin, or immunoprecipitation buffer II [10 mM Tris/HCl, pH 7.4, containing 0.5 % (v/v) Nonidet P40, 150 mM NaCl, 0.02 % (w/v) NaN_3, 1 mM PMSF and 1 mM disodium EDTA], which was used with lysates prepared with 1 % (v/v) Nonidet P40. The agarose beads were then resuspended at 1:1 (v/v) in the appropriate buffer.} Supernatants were removed after pelleting the samples at 11000 ***g*** for 4 min in an Eppendorf Microfuge. The pellets were washed once with 100 μl of Dulbecco's PBS and the supernatants for each sample were pooled. The samples were then incubated with the anti-(human small-intestinal mucin) antibody for 2 h at room temperature, followed by the addition of Protein A–agarose and overnight incubation at 4 °C. Other antibodies, namely anti-CRT, anti-CLN, anti-MRP and anti-MUC5AC, replaced the anti-(human small-intestinal mucin) antibody in some experiments. With the MUC5AC antibody, goat anti-mouse IgG–agarose was used instead of Protein A–agarose. The samples were then centrifuged at 11000 ***g*** for 4 min in an Eppendorf Microfuge and the pellets were washed three times with 1 ml of the appropriate immunoprecipitation buffer and once with the buffer minus detergent. The pellets were then suspended in 50 μl of SDS/PAGE sample buffer [containing 2 % (w/v) SDS, with or without 5 % (v/v) 2-mercaptoethanol] and boiled for 3 min.

In one experiment, the CRT and CLN immunoprecipitates were incubated for 2 h at room temperature in 200 μl of 50 mM Tris/HCl buffer, pH 7.5, containing 150 mM NaCl, 1 % Nonidet P40, 0.5 % sodium deoxycholate, 0.1 % SDS, Complete™ (one tablet /10 ml) and pepstatin (10 μg/ml). The samples were then pelleted and the supernatants reprecipitated with anti-MUC2 and Protein A–agarose.

Electrophoresis and autoradiography

Samples were usually analysed by SDS/PAGE according to [29], using a 3 %-acrylamide stacking gel and a 4 %-acrylamide running gel (Miniprotean II; Bio-Rad). Prestained high-molecular-mass standards (Bio-Rad) and rat L2 yolk-sac-tumour laminin (giving bands at 200 and 400 kDa when run under reducing conditions) (Calbiochem) were used as molecular-mass markers. In some instances SDS/PAGE was performed using 3–12 % gradient CAP™ gels (ICN Pharmaceuticals Inc.; Costa Mesa, CA, U.S.A.). After electrophoresis all gels were fixed in a solution containing 30 % (v/v) methanol, 10 % (v/v) acetic acid and 10 % (w/v) trichloroacetic acid for 1 h at room temperature, then washed for 10 min in distilled water and dried. The gels were then exposed to Kodak Biomax MR 1 film (exposure time ranged from 1 to 14 days). Films were scanned on an Agfa Arcus II scanner and images were made using Adobe Photoshop 4.0 and printed using a Kodak dye-sublimation printer.

Western blotting

Cell lysate proteins were separated by SDS/PAGE (3–12 % gradient gels) and transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) using a Miniblot Transfer Kit (Bio-Rad). Membranes were blocked with 3 % (w/v) BSA (Sigma, A-7030) in 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl and 0.05 % (v/v) Tween 20 (TBST) for 1 h at room temperature. The membranes were then incubated at 4 °C overnight in either anti-CRT or anti-CLN (dilution 1:1000) and washed with TBST. Goat anti-rabbit IgG–alkaline phosphatase conjugate (Bio-Rad) at a dilution of 1:3000 was added and the membranes were incubated for 1 h at room temperature, washed with TBST and bound antibody was detected colorimetrically using an alkaline phosphatase substrate (Bio-Rad).

Endo H digestion

Mucin antigens were immunoprecipitated from 100 μl aliquots of cell lysate with either the anti-(human small-intestinal mucin) or the anti-CRT antibody as described above. After washing the Protein A–agarose pellets with buffer minus detergent, they were suspended in 50 μl 0.1 M sodium acetate buffer, pH 5.5, containing 0.1 % (w/v) SDS and 1 mM PMSF with or without endo H (16 m-unit/ml) and incubated for 17 h at 37 °C. At the end of the incubation period, the samples were pelleted at 11 000 ***g*** for 4 min in an Eppendorf Microfuge. The pellets were then suspended in 50 μl reducing SDS/PAGE sample buffer and boiled for 3 min.

RESULTS

LS180 and HT29/A1 cells synthesize homo-oligomers of MUC2 and MUC5AC

Initial experiments were performed to demonstrate that both monomeric precursors and oligomers of MUC2 and MUC5AC were being synthesized during the timeframe studied, and also to determine if homo- and/or hetero-oligomers were being produced by the two cell lines. Figure 1 shows results from experiments in which cells were radiolabelled with [^{35}S]Promix for 15 min, chased for 0, 0.5 and 2 h, and cell extracts immunoprecipitated by antibody (MRP) specific for MUC2 or antibody specific for MUC5AC. When analysed by SDS/PAGE under non-reducing conditions (Figure 1a, lanes 1–3), band A and a more slowly migrating band B of MUC2 mucin from LS180 cells were seen at the earliest time, whereas only band B was present at later times. Under reducing conditions (Figure 1a, lanes 4–6) only band A (apparent molecular mass about 600000 Da) was present at all three time points. From previous studies [11] it was recognized that band A is a monomeric precursor of MUC2 and band B represents disulphide-linked oligomers of band A. Identical results were obtained using an antibody to human small-intestinal mucin, which recognizes both immature and mature mucin, in place of the MRP antibody [11], and this antibody therefore was used to detect MUC2 in all subsequent experiments. When HT29/A1 cell extracts were immunoprecipitated with anti-MUC2, bands A and B were also present under non-reducing conditions and only band A under reducing conditions (results not shown). Thus the two MUC2 bands were present in both cell lines at the same time points. On immunoprecipitation of HT29/A1 cell extracts with anti-MUC5AC, a mucin band C was observed under non-reducing conditions immediately after the pulse and was gradually replaced by a band D of greater molecular mass at later times (Figure 1b, lanes 1–3). On reduction, only band C (apparent molecular mass approx. 500000 Da) was present at each time point (Figure 1b, lanes 4–6) indicating that band D was a disulphide-bonded oligomer of band C. Similar results were obtained when LS180 cells instead of HT29/A1 cells were studied with anti-MUC5AC (results not shown). Thus the antibodies to MUC2 and MUC5AC recognized mucins of different molecular mass and both mucins were synthesized in

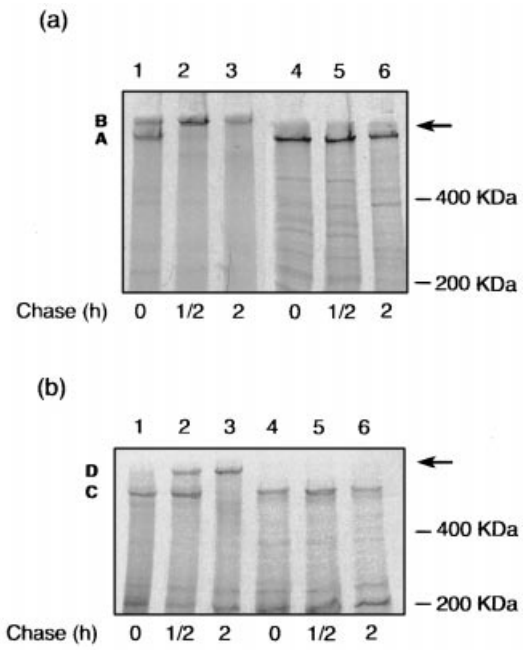

Figure 1 Homo-oligomers of MUC2 and MUC5AC are synthesized in LS180 and HT29/A1 cells

Autoradiograms of SDS/polyacrylamide gels showing immunoprecipitations from cell extracts. Cells were pulsed with [^{35}S]Promix for 15 min, chased for the times indicated and then lysed in digitonin-containing buffer (as described in the Materials and methods section). (**a**) LS180 cell extracts treated with anti-MRP (MUC2) antibody; (**b**) HT29/A1 cell extracts with anti-MUC5AC antibody. Lanes 1–3 are non-reducing SDS/PAGE and 4–6, reducing SDS/PAGE. The arrowhead indicates the junction of the 3% stacking with the 4% separating gel. Bands A, B, C and D are described in the Results section.

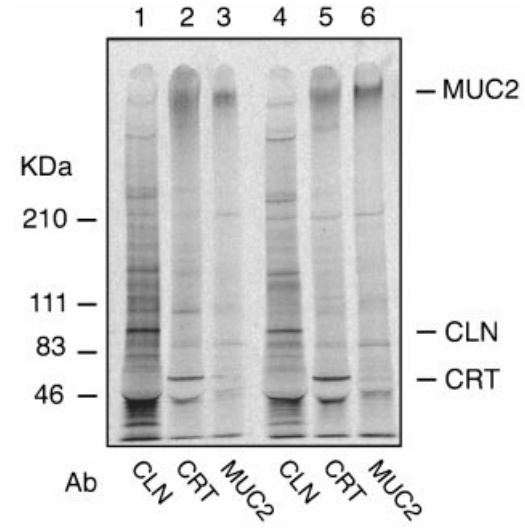

Figure 2 Antisera (Ab) to CRT and CLN co-precipitate various proteins from radiolabelled LS180 cell extracts

LS180 cells were pulsed with [^{35}S]Promix for 15 min (lanes 1–3) or pulsed for 15 min and chased for 0.5 h (lanes 4–6) and then lysed in digitonin-containing buffer. Immunoprecipitations of cell extracts with anti-CLN, anti-CRT or anti-MUC2 antisera were performed for both time points and samples analysed by reducing SDS/PAGE using a 3–12% gradient gel and autoradiography. Lanes 1 and 4, anti-CLN; 2 and 5, anti-CRT and 4 and 6, anti-MUC2. The positions of MUC2, CLN and CRT on the gels are indicated.

each of the cell lines studied. Significantly, there was no evidence of hetero-oligomer formation in these cells, since only one band representing a monomer was obtained under reducing conditions in each case. Therefore only homo-oligomers of MUC2 and MUC5AC are produced by LS180 and HT29/A1 cells, allowing us to examine the chaperone requirements of each mucin during synthesis.

Interaction of CRT and CLN with MUC2 mucin precursors in LS180 cells

Since N-glycans can play a role in ER synthesis and we, and others, have noted that initial N-glycosylation is important for efficient oligomerization of both MUC2 [11,13,16] and MUC5AC [17], we looked for involvement of the chaperones CRT and CLN, which recognize mono-glucosylated N-glycans, during mucin synthesis in the ER. Preliminary experiments (results not shown), in which the proteins of LS180 and HT29/A1 cell lysates were separated by SDS/PAGE followed by Western blotting with the antibodies to CRT and CLN, resulted in blots with specific immunoreactive bands at 60 kDa, typical of CRT [30], and 90 kDa, typical of CLN [31].

Experiments were then carried out to determine if the anti-CLN and anti-CRT antisera were capable of co-precipitating radiolabelled proteins from lysates of [^{35}S]Promix-labelled LS180 cells. The gradient gel shown in Figure 2 demonstrates that anti-CLN immunoprecipitated labelled CLN (lanes 1 and 4) and, similarly, anti-CRT immunoprecipitated labelled CRT (lanes 2 and 5) and that both antisera also co-precipitated a number of other labelled proteins at the two time points studied. These co-precipitated proteins were not identical for the two antisera (compare lanes 1 and 2, and 4 and 5). As a control, immunoprecipitation was also performed with anti-MUC2 (Figure 2, lanes 3 and 6). A broad band of MUC2 was detected near the top of the gel and a similar band was present in the anti-CRT co-precipitated material (lanes 2 and 5). A minor band in the same region was present when anti-CLN replaced anti-CRT (lanes 1 and 4).

To determine if either CRT or CLN specifically functioned as chaperones during MUC2 synthesis several experiments were then carried out. Co-precipitation of radiolabelled mucin with each chaperone was performed at various times during pulse–chase labelling followed by analysis on SDS/PAGE using a 3% stacking and a 4% separating gel. Experiments using LS180 cells (Figures 3a and 3b, lanes 1–6) showed anti-CRT co-precipitated labelled proteins having the same mobilities as MUC2 bands A and B on non-reducing and reducing SDS/PAGE. Immediately after the 15 min pulse anti-CRT co-precipitated bands with the same mobility as A and B, and similar to the precipitation of labelled MUC2 with anti-MUC2 (Figure 3a, lanes 1 and 4). In contrast, band B was co-precipitated weakly after 0.5 h and negligibly after 2 h of chase when compared with labelled MUC2 precipitated with anti-MUC2 antibody (Figure 3a, lanes 2 and 5, and lanes 3 and 6). A labelled band of low intensity in the position of monomeric MUC2 (A) was co-precipitated with the anti-CLN antibody at 0 and 0.5 h of chase, but was barely detectable after 2 h (Figures 3a and 3b, lanes 8–10).

To confirm that the chaperone antibodies had precipitated MUC2, anti-CRT and anti-CLN co-precipitation of ^{35}S-labelled protein from the cells was repeated and was followed by elution of labelled protein from the Protein A–agarose pellets. Each eluate was then incubated with anti-MUC2 (as described in the

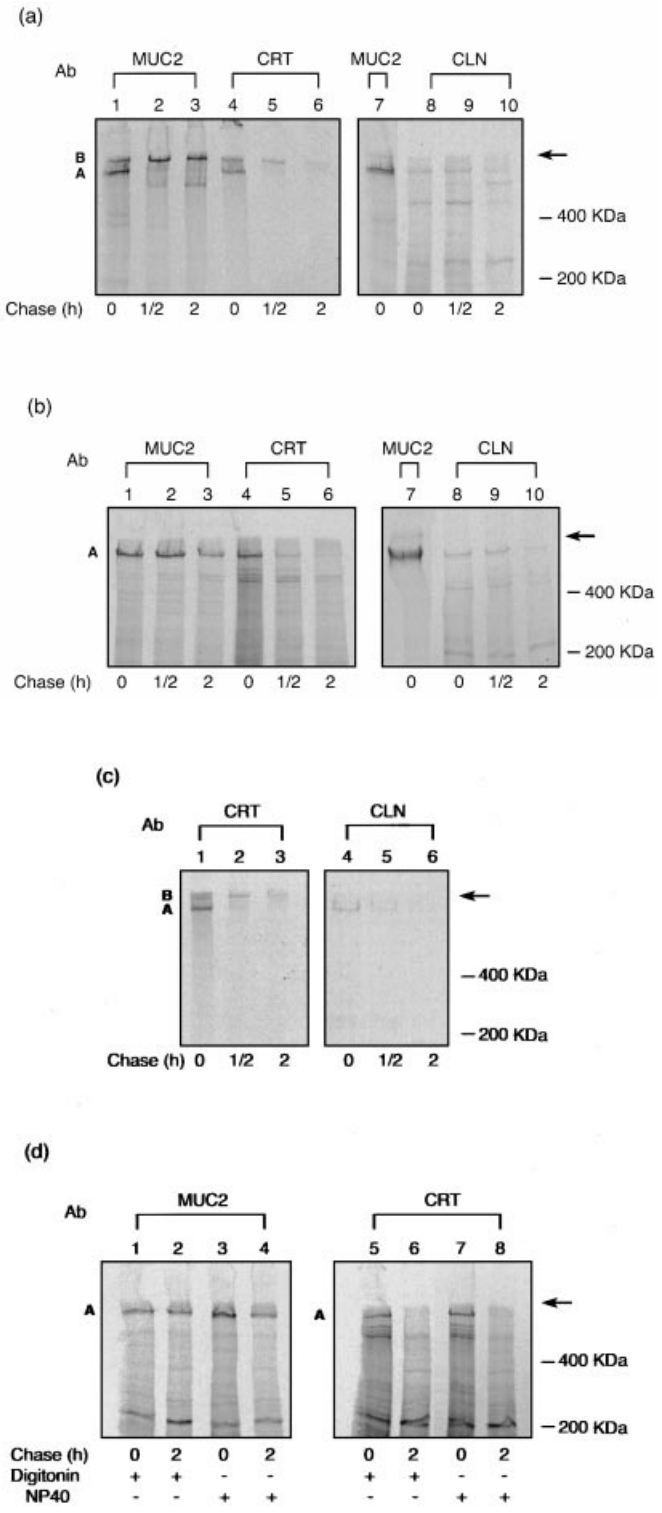

Figure 3 Interaction of CRT and CLN with MUC2 mucin precursors in LS180 cells

Pulse–chase was performed as described in the legend to Figure 1. (**a**) Non-reducing SDS/PAGE and (**b**) reducing SDS/PAGE of LS180 cell extracts treated with anti-MUC2 antibody (Ab) (lanes 1, 2, 3 and 7), anti-CRT antibody (lanes 4–6) and anti-CLN antibody (lanes 8–10). (**c**) Non-reducing SDS/PAGE in an experiment in which co-precipitation with anti-CRT or anti-CLN was followed by elution of the precipitate and re-precipitation with anti-MUC2. Co-precipitation was with anti-CRT (lanes 1–3) or with anti-CLN (lanes 4–6). (**d**) Comparison of immunoprecipitation experiments with anti-CRT and anti-MUC2 after lysis of the cells with buffer containing either 1% digitonin or 1% Nonidet P40 (NP40).

Materials and methods section) and labelled MUC2 was recovered from the CRT immunoprecipitate after the 15 min pulse (Figure 3c, lanes 1–3). Less was recovered after 0.5 h of chase and there was virtually none after 2 h of chase. Small amounts of labelled MUC2 were also recovered from the CLN immunoprecipitates at 0 and 0.5 h of chase (Figure 3c, lanes 4–6). These results confirm that the labelled proteins co-precipitated by anti-CRT and anti-CLN (Figures 3a and 3b) were indeed MUC2. The associations between CRT and MUC2 and between CLN and MUC2 were transient, since they had largely disappeared by 2 h of chase at a time when labelled MUC2 could still be precipitated from the cells by anti-MUC2 (Figures 3a and 3b, lanes 3 and 6). Co-precipitation of MUC2 with anti-CRT still took place when the LS180 cells were lysed in buffer containing a stronger detergent, 1% Nonidet P40, in place of 1% digitonin (Figure 3d) and the pattern of results was the same as shown in Figure 3(b). In contrast, no co-precipitation of MUC2 with CLN was detected after cell lysis in Nonidet P40-containing buffer (result not shown). Thus the transient interaction between CRT and MUC2 was more stable than that between CLN and MUC2.

CRT and CLN do not interact with MUC5AC in HT29/A1 cells

The cells were pulsed with [^{35}S]Promix for 15 min, chased for 0, 0.5 or 2 h and the cell extracts were precipitated with antibodies to either MUC5AC, MUC2, CRT or CLN. Figure 4 shows that MUC2 and MUC5AC were precipitated by their respective antibodies from HT29/A1 cells (lanes 1–3 and lanes 7–9), and anti-CRT co-precipitated MUC2 in a transient manner (lanes 4–6), as shown previously in LS180 cells (Figure 3b, lanes 4–6). In contrast, there was no co-precipitation of MUC5AC with the CRT antibody at any time point (Figure 4, lanes 4–6). Similar results were obtained using LS180 cells (Figure 3a, lanes 4–6). In addition, no binding of either MUC2 or MUC5AC to CLN was initially detected, but when the gels were exposed to film for twice the length of time (6 days instead of 3 days) very faint bands in the region of MUC2, but not MUC5AC, were detected (Figure 4, lanes 10–12). These bands were present at 0 and 0.5 h of chase. Thus the findings in HT29/A1 cells for MUC2 and MUC5AC co-precipitated with anti-CLN are in agreement with results obtained using the LS180 cells (Figure 3b, lanes 8–10). As the interaction between CRT and MUC2 was stronger than that between CLN and MUC2 in both cell lines (Figures 3 and 4) we decided to look at the binding to CRT in greater detail.

Treatment of labelled MUC2 immunoprecipitates with Endo H

We have shown previously [11] that initial oligomerization of MUC2 in LS180 cells occurs before a monensin-sensitive step in synthesis, i.e. before the medial Golgi, and recently others have reported similar findings for both MUC2 and MUC5AC in LS174T cells and have demonstrated that oligomerization occurs in the ER [13,16,17]. Since our experiments suggested that CRT dissociates from MUC2 after oligomerization (Figure 3), it is most likely acting as a chaperone in the ER. To test this hypothesis, we treated radiolabelled MUC2, which had been either precipitated with anti-MUC2 at 0, 0.5 h and 2 h chase or co-precipitated with CRT at 0 and 0.5 h chase, with endo H. In this experiment no co-precipitation of MUC2 with CRT was found after a 2 h chase. The mucin was sensitive to endo H at the 0 and 0.5 h time points (Figure 5, lanes 4, 5, 8 and 10) but was resistant after 2 h of chase (lane 6). Thus MUC2 had reached the

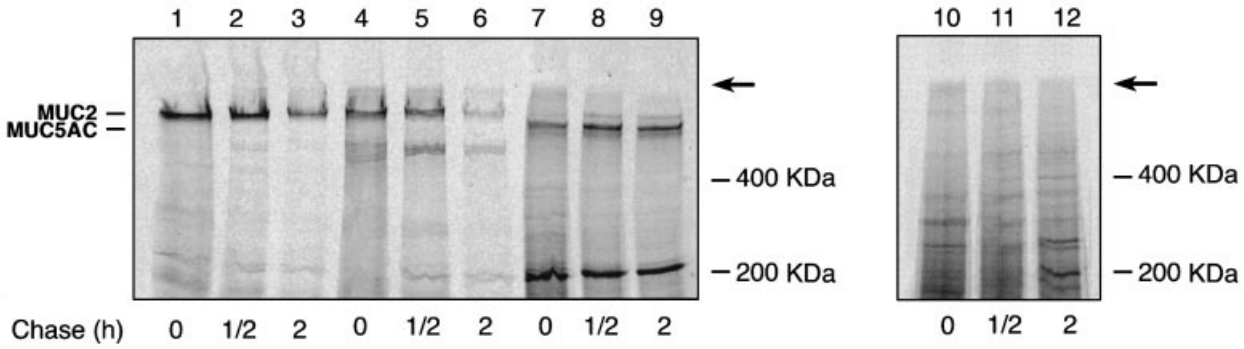

Figure 4 Lack of association of CRT and CLN with MUC5AC mucin precursors in HT29/A1 cells

Cells were pulsed with [^{35}S]Promix for 15 min and chased for 0, 0.5 and 2 h. Reducing SDS/PAGE of HT29/A1 cell extracts treated with anti-MUC2 antibody (lanes 1–3), anti-CRT antibody (lanes 4–6), anti-MUC5AC antibody (lanes 7–9) and anti-CLN antibody (lanes 10–12).

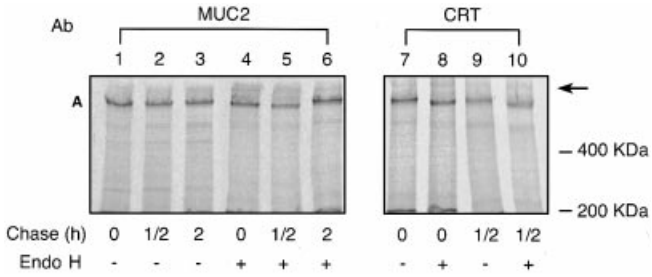

Figure 5 Endo H treatment of LS180 mucin

LS180 cells were pulsed with [^{35}S]Promix for 15 min and chased for 0, 0.5 and 2 h. Cell extracts were immunoprecipitated with either anti-MUC2 or anti-CRT antibody (Ab), the immunoprecipitates incubated with or without endo H (as described in Materials and methods section) and analysed by reducing SDS/PAGE and autoradiography. Lanes 1, 2, 3, 7 and 9, without endo H; lanes 4, 5, 6, 8 and 10, with endo H.

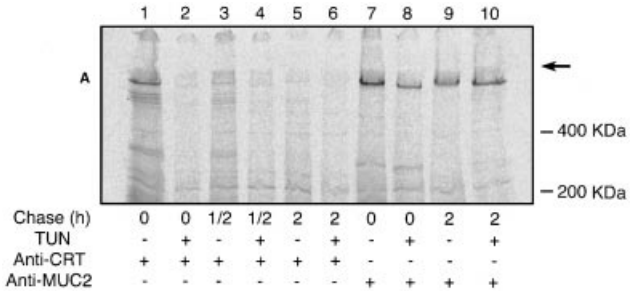

Figure 6 Effect of TUN on the MUC2-CRT interaction

LS180 cells were preincubated for 45 min, pulsed for 15 min and chased for 0, 0.5 or 2 h in the presence or absence of TUN (20 μg/ml). Immunoprecipitations of cell extracts with either the anti-CRT antibody or the anti-MUC2 antibody were performed for each time point and the samples analysed by reducing SDS/PAGE and autoradiography. Lanes 1–6, anti-CRT antibody; lanes 7–10, anti-MUC2 antibody. Lanes 1, 3, 5, 7 and 9 without TUN; lanes 2, 4, 6, 8 and 10 with TUN.

Golgi by 2 h of chase and no longer contained N-glycans rich in mannose. As CRT bound to the mucin when it was endo H-sensitive, the binding is likely to occur in the ER.

Effect of TUN on the CRT–MUC2 interaction

LS180 cells were treated with TUN, an inhibitor of N-glycosylation, to see if the CRT–MUC2 interaction was affected. Figure 6 (lanes 7–10) shows that monomeric MUC2 migrated faster on reducing SDS/PAGE in the presence of TUN, which was consistent with an inhibition of N-glycosylation. TUN treatment also inhibited the interaction of MUC2 with CRT (Figure 6, lanes 2, 4 and 6) compared with that of untreated controls (Figure 6, lanes 1, 3 and 5). This was not due to a depletion of CRT, since Western blotting of LS180 cell extracts with anti-CRT demonstrated that significant amounts of CRT were present at all time points (results not shown). Inhibition of CRT association with MUC2 by TUN therefore suggests that N-glycans on the mucin are necessary for the chaperone–mucin interaction. This is not surprising because CRT binding to a number of proteins is dependent on the presence of mono-glucosylated N-oligosaccharides generated in the ER after trimming of the core glycan by glucosidases I and II [22,24]. Accordingly, we attempted to identify the stage of glycosylation processing of MUC2 that was required for CRT interaction.

Effect of CAS on the CRT–MUC2 interaction

CAS is known to inhibit glucosidase I, an enzyme that removes glucose residues from $Glc_3Man_9GlcNAc$ oligosaccharides that are attached to nascent polypeptide chains as they enter the ER [32]. This inhibitor prevents CLN and CRT from binding to a number of newly-synthesized proteins, since the Glc_1Man_9 GlcNAc glycan required for binding by the chaperones is not produced [33]. Figures 7(a) and 7(b) show the effect of CAS on the CRT–MUC2 interaction at various times. Surprisingly the co-precipitation of MUC2 by the CRT antibody was not inhibited by CAS and, in fact, it was sustained for up to 4 h of chase. Figure 7(a) also shows that CRT remained associated with oligomeric MUC2 (band B) at later chase times, and anti-CRT co-precipitated other labelled proteins (bands between 400 and 600 kDa) from cell lysates. In addition, the secretion of MUC2 was inhibited by approx. 60 % after a 4 h chase by CAS treatment (results not shown). Treatment of the CRT-co-precipitated MUC2 with endo H at the time points shown in Figures 7(a) and 7(b) demonstrated that the mucin was sensitive to the enzyme at up to 4 h of chase (Figure 7c). Similar results were obtained using the HT29/A1 cells (results not shown). These observations imply that, in the presence of CAS, immature MUC2 was not being processed correctly and remained bound to CRT in the ER. In

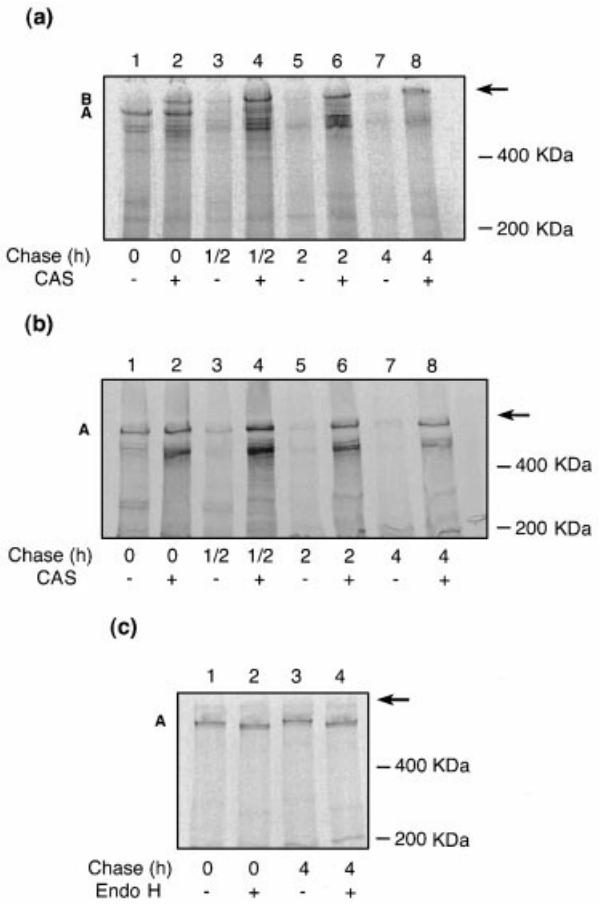

Figure 7 Effect of CAS on the MUC2–CRT interaction

LS180 cells were preincubated for 45 min, pulsed for 15 min and chased for 0, 0.5 or 2 h in the presence or absence of CAS (1 mM). Immunoprecipitations of cell extracts with anti-CRT were performed for each time point and the samples analysed by SDS/PAGE and autoradiography. (**a**) Non-reducing SDS/PAGE; (**b**) reducing SDS/PAGE. Lanes 1–6, anti-CRT antibody; lanes 7–10, anti-MUC2 antibody. Lanes 1, 3, 5, and 7, without CAS; lanes 2, 4, 6 and 8, with CAS. (**c**) Reducing SDS/PAGE of MUC2 co-precipitated by anti-CRT in the presence of CAS and treated with or without endo H. Lanes 1 and 3, without endo H; lanes 2 and 4, with endo H.

the above experiments, the LS180 cells were treated with 1 mM CAS, a concentration that is commonly used in chaperone studies [22,24,34]. However, since 1 mM CAS strongly impaired MUC2 processing and secretion, the experiments were repeated after lowering the CAS concentration from 1 to 0.56 mM, the minimum level required to inhibit glucosidase I almost completely, or after decreasing the preincubation time with CAS from 45 to 10 min. In both cases, results for cellular MUC2 similar to those in Figures 5(a) and 5(b) were obtained, but MUC2 secretion was less inhibited (results not shown). Moreover, when CAS was replaced by 1 mM 1-deoxynojirimycin, another glucosidase inhibitor, MUC2 still remained bound to CRT after a 2 h chase (results not shown). Thus both inhibitors caused retention of MUC2 precursors in the ER, where they remained associated with CRT.

DISCUSSION

The observation that only homo-oligomers of MUC2 and MUC5AC are synthesized in the ER of LS180 and HT29/A1 cells (Figure 1) is in accord with recently published results using the colonic-tumour-cell line LS174T [13,16,17]. The authors of these papers also strongly suggest that LS174T oligomers are in fact dimers. When we initiated our studies it was not known if chaperones are involved during the synthesis of large mucin molecules. In general, however, a quality-control apparatus in the ER made up of molecular chaperones and folding enzymes ensures the functional integrity of secretory proteins and regulates their transport. Our results demonstrate that CRT is involved as a chaperone during the ER synthesis of MUC2 in both HT29/A1 and LS180 cells. CRT bound transiently to MUC2 mucin precursors (Figures 3 and 4), but once the precursors had completely oligomerized and moved from the ER to the Golgi, as shown by the development of resistance to endo H (Figure 5), CRT was no longer associated with the mucin.

A weak interaction between CLN and MUC2 was detected in LS180 cells (Figure 3) at the same chase times when CRT bound strongly to MUC2. This could be due to the fact that CLN is membrane-bound and CRT is luminally oriented. There are a number of examples where immature proteins bind to both chaperones and others where binding is exclusive to one or other chaperone [35–37]. In some instances the binding to CRT and CLN appears to be determined by the assembly state of the target molecule, for example during the biosynthesis of myeloperoxidase [38] and Factor VIII [39] and also during the assembly of MHC class I molecules [40]. Since CRT associated with both the MUC2 mucin monomer and oligomer and CLN appeared to bind the monomer only (Figure 3), CRT and CLN may perhaps be participating in a co-ordinated manner during protein synthesis in the ER. Precise roles for CLN and CRT during MUC2 synthesis might possibly be determined by studying binding at a larger series of time points during folding and oligomerization in the ER. In fact, we have recently found that CRT is already bound to MUC2 in LS180 cells after a 5 min pulse of [^{35}S]Promix and that the complex is present at 1 h and 1.5 h chase times. Also, it would be of interest to study the involvement of CRT and CLN during the disulphide-mediated dimerization of rat Muc2 C-terminus peptides in transfected cells as described in [10].

Despite some structural similarity to MUC2, no interaction between MUC5AC and either CRT or CLN at the same time points could be detected (Figures 3 and 4). Thus MUC2 as an immature unfolded glycoprotein may contain some unique features which are recognized by CRT for chaperone binding at the particular time points studied. For example it is known from studies with human class I MHC proteins with altered N-glycosylation that both the number and location of N-linked oligosaccharides within proteins can influence their folding and interaction with CRT [41]. Recent studies using transfected Chinese-hamster ovary (CHO) cells have examined the roles of CRT and CLN during synthesis of the blood-coagulation factors (Factors V and VIII) [39]. These glycoproteins have a conserved domain organization of A1-A2-B-A3-C1-C2 and share about 35% amino acid identity in their A and C domains. Their B domains have little homology but contain a large number of potential N-glycosylation sites and are probably the initial binding location of CRT and CLN. Even with these similarities, Factor V and Factor VIII were found to have different requirements for CRT and CLN during their passage through the ER. Perhaps a similar situation exists for MUC2 and MUC5AC. Another possible reason for CRT associating with the colonic mucin MUC2, but not with the gastric-type mucin MUC5AC, at the times studied could arise from the synthesis of each mucin by different populations of cells in the LS180 and HT29/A1 cultures, each population having its own programme of chaperone interactions during ER processing.

Inhibition of the CRT-MUC2 binding by TUN (Figure 6) suggested that N-glycosylation was important for the CRT interaction and that monoglucosylated N-glycans might be involved. The unexpected finding that CAS or 1-deoxynojirimycin treatment to inhibit glucosidases I and II did not destroy the CRT-MUC2 association (Figure 7) could be due to a number of factors. In the presence of glucosidase inhibitors CRT may bind only to polypeptide. For example, direct binding of CRT to the peptide KLGFFKR, which is a highly conserved region in the α subunit of integrins [42], and binding, via its P domain, to PDI [43] has been reported. In addition it has been shown [39] that CAS and 1-deoxynojirimycin inhibited the association of CLN but not of CRT to Factor VIII in CHO cells. Thus monoglucosylated high-mannose structures on MUC2 may not be a prerequisite for CRT binding. Another possible explanation of our findings is that CAS is known to inhibit other enzymes in addition to glucosidase I, and as a consequence has a number of effects on cells, including inhibition of protein secretion in some cases (see [32] for a review). It is not known how many molecules of CRT are available to associate with one molecule of immature MUC2 at any one time, and inhibitor treatment may alter this ratio. The observation (Figure 7) that CRT remained bound to MUC2 longer in the presence of CAS and that the MUC2 remained sensitive to endo H suggests that CRT was continuing to bind to misfolded mucin aggregates in the ER. This type of behaviour has been described [44] for another chaperone, PDI, in situations where unfolded protein is in excess, and may be a mechanism to retain unfolded proteins or to prevent their degradation in stressed cells. However, when PDI is in excess, proteins are folded and assembled properly [44]. Thus changes in cellular concentration of chaperone relative to immature protein may account for the differences in CRT–MUC2 association in LS180 cells in the presence or absence of CAS. It has also been recently reported that CAS treatment of CHO cells stably expressing the human insulin receptor resulted in protein misfolding and premature homodimerization [45]. There may be a similar effect of CAS on MUC2 synthesis in LS180 cells (Figure 7a). It appears that, for high-efficiency ER maturation of proteins, CRT and/or CLN should promote proper folding before oligomerization, and perhaps monoglucosylated N-glycans are necessary for this sequence of events.

It is currently believed that newly synthesized glycoproteins interact with a network of ER-resident chaperones which may act as a matrix that binds early assembly intermediates and prevents premature exit from the ER [46]. Thus quality control appears to involve multiple interactions with ER chaperones [47]. Our experiments to look at chaperone binding to gastrointestinal mucins in colonic tumour cells are the first to provide information on the roles of CRT and CLN during synthesis of a large secretory mucin (MUC2). Much work remains to be done to describe in detail the interactions of not only CRT and CLN, but of other chaperones, such as BiP and PDI, during the ER synthesis of gastrointestinal mucins.

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REFERENCES

1 Tytgat, K. M. A. J., Büller, H. A., Opdam, F. J. M., Kim, Y. S., Einerhand, A. W. C. and Dekker, J. (1994) Gastroenterology **107**, 1352–1363
2 Klomp, L. W. J., van Rens, L. and Strous, G. J. (1994) Biochem. J. **304**, 693–698
3 Forstner, J. F. and Forstner, G. (1994) Physiology of the Gastrointestinal Tract, 3rd. edn. (Johnson, L. R., ed.), pp. 1255–1283, Raven Press, New York
4 Van Klinken, B. J.-W., Dekker, J., Büller, H. A. and Einerhand, A. W. C. (1995) Am. J. Physiol. **269**, G613–627
5 Li, D., Gallup, M., Fan, N., Szymkowski, D. E. and Basbaum, C. B. (1998) J. Biol. Chem. **273**, 6812–6820
6 Gum, Jr., J. R., Hicks, J. W., Toribara, N. W., Siddiki, B. and Kim, Y. S. (1994) J. Biol. Chem. **269**, 2440–2446
7 Van de Bovencamp, J. H. B., Hau, C. M., Strous, G. J. A. M., Büller, H. A., Dekker, J. and Einerhand, A. W. C. (1998) Biochem. Biophys. Res. Commun. **245**, 853–859
8 Wagner, D. D. (1990) Annu. Rev. Cell Biol. **6**, 217–246
9 Dong, Z., Thoma, R. S., Crimmens, D. L., McCourt, D. W., Tuley, E. A. and Sadler, J. E. (1994) J. Biol. Chem. **269**, 6753–6758
10 Bell, S. L., Khatri, I. A., Xu, G. and Forstner, J. F. (1998) Eur. J. Biochem. **253**, 123–131
11 McCool, D. J., Forstner, J. F. and Forstner, G. G. (1994) Biochem. J. **302**, 111–118
12 Van Klinken, B. J.-W., Oussoren, E., Weenink, J.-J., Strous, G. J., Büller, H. A., Dekker, J. and Einerhand, A. W. C. (1996) Glycoconjugate J. **13**, 757–768
13 Van Klinken, B. J.-W, Einerhand, A. W. C., Büller, H. A. and Dekker, J. (1998) Glycobiology, **8**, 67–75
14 Specian, R. D. and Oliver, M. G. (1991) Am. J. Physiol. **260**, C183–C193
15 McCool, D. J., Forstner, J. F. and Forstner, G. G. (1995) Biochem. J. **312**, 125–133
16 Asker, N., Axelsson, M. A. B., Oloffson, S.-O. and Hansson, G. C. (1998) J. Biol. Chem. **273**, 18857–18863
17 Asker, N., Axelsson, M. A. B., Oloffson, S.-O. and Hansson, G. C. (1998) Biochem. J. **335**, 381–387
18 Gething, M.-J. and Sambrook, J. (1992) Nature (London) **355**, 33–45
19 Ruddon, R. W. and Bedows, E. (1997) J. Biol. Chem. **272**, 3125–3128
20 Hammond, C. and Helenius, A. (1995) Curr. Opin. Cell Biol. **7**, 523–529
21 Krause, K.-H. and Michalak, M. (1997) Cell **88**, 439–443
22 Nauseef, W. M., McCormick, S. J. and Clark, R. A. (1995) J. Biol. Chem. **270**, 4741–4747
23 Ware, F. E., Vassilakos, A., Peterson, P. A., Jackson, M. R., Lehrman, M. A. and Williams, D. B. (1995) J. Biol. Chem. **270**, 4697–4704
24 Hebert, D. N., Foellmer, B. and Helenius, A. (1996) EMBO J. **15**, 2961–2968
25 Vassilakos, A., Michalak, M., Lehrman, M. A. and Williams, D. B. (1998) Biochemistry **37**, 3480–3490
26 Gum, J. R., Bird, J. W., Hicks, J. W., Toribara, N. W., Lamport, D. T. A. and Kim, Y. S. (1989) J. Biol. Chem. **264**, 6480–6487
27 Sotozono, M., Okada, Y., Sasagawa, T., Nakatou, T., Yoshida, A., Yokoi, T., Kubota, M. and Tsuji, T. (1996) J. Immunol. Methods **192**, 87–96
28 Kreusel, K.-M., Fromm, M., Schultzke, J. and Hegel, U. (1991) Am. J. Physiol. **261**, C574–C582
29 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
30 Michalak, M., Milner, R. E., Burns, K. and Opas, M. (1992) Biochem. J. **285**, 681–692
31 Ou, W.-J., Cameron, P. H., Thomas, D. Y. and Bergeron, J. J. M. (1993) Nature (London) **364**, 771–776
32 Kaushal, G. P. and Elbein, A. D. (1994) Methods Enzymol. **230**, 316–329
33 Hebert, D. N., Foellmer, B. and Helenius, A. (1995) Cell **81**, 425–433
34 Vassilakos, A., Cohen-Doyle, M. F., Peterson, P. A., Jackson, M. R. and Williams, D. B. (1996) EMBO J. **15**, 1495–1506
35 Peterson, J. R., Ora, A., Van, P. N. and Helenius, A. (1995) Mol. Biol. Cell **6**, 1173–1184
36 Van Leeuwen, J. E. M. and Kearse, K. P. (1996) J. Biol. Chem. **271**, 25345–25349
37 Helenius, A., Trombetta, E. S., Hebert, D. N. and Simons, J. F. (1997) Trends Cell Biol. **7**, 193–200
38 Nauseef, W. M., McCormick, S. J. and Goedken, M. (1998) J. Biol. Chem. **273**, 7107–7111
39 Pipe, S. W., Morris, J. A., Shah, J. and Kaufman, R. J. (1998) J. Biol. Chem. **273**, 8537–8544
40 Harris, M. R., Yu, Y. Y. L., Kindle, C. S., Hansen, T. H. and Solheim, J. C. (1998) J. Immunol. **160**, 5404–5409
41 Zhang, Q. and Salter, R. D. (1998) J. Immunol. **160**, 831–837
42 Rojiani, M. V., Finlay, B. B., Gray, V. and Dedhar, S. (1991) Biochemistry **30**, 9859–9866
43 Baksh, S., Burns, K., Andrin, C. and Michalak, M. (1995) J. Biol. Chem. **270**, 31338–31344
44 Gilbert, H. F. (1997) J. Biol. Chem **272**, 29399–29402
45 Bass, J., Chiu, G., Argon, Y. and Steiner, D. F. (1998) J. Cell Biol. **141**, 637–646
46 Tatu, U. and Helenius, A. (1997) J. Cell Biol. **136**, 555–565
47 Zhang, J.-X., Braakman, I., Matlack, K. E. S. and Helenius, A. (1997) Mol. Biol. Cell **8**, 1943–1954

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