The oligomerization of a family of four genetically clustered human gastrointestinal mucins

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Mucins are synthesized and secreted by many epithelia. They are complex glycoproteins that offer cytoprotection. In their functional configuration, mucins form oligomers by a biosynthetic process that is poorly understood. A family of four human gastrointestinal mucin genes (MUC2, MUC5AC, MUC5B, and MUC6) is clustered to chromosome 11p15.5. To study oligomerization of these related mucins, we performed metabolic labeling experiments with [35S]amino acids in LS174T cells, and isolated mucin precursors by specific immunoprecipitations that were analyzed on SDS–PAGE. Each of the precursors of MUC2, MUC5AC, MUC5B, and MUC6 formed a single species of disulfide-linked homo-oligomer within 1 h after pulse labeling. Based on apparent molecular masses, these oligomeric precursors were most likely dimers. Inhibition of vesicular RER-to-Golgi transport, with brefeldin A and CCCP, did not affect the dimerization of MUC2 precursors, localizing dimerization to the RER. O-Glycosylation of MUC2 followed dimerization. Inhibition of N-glycosylation by tunicamycin retarded, but did not inhibit, dimerization, indicating that N-glycans play a role in efficient dimerization of MUC2 precursors. Based on sequence homology, the ability of MUC2, MUC5AC, MUC5B and MUC6 to dimerize most likely resides in their C-terminal domains. Thus, the RER-localized dimerization of secretory mucins likely proceeds by similar mechanisms, which is an essential step in the formation of the human gastrointestinal mucus-gels.

Key words: biosynthesis/LS174T cell line/mucins/ oligomerization

Introduction

Mucus comprises a visco-elastic gel that covers and protects the mucosal surface of the gastrointestinal tract (Forstner and Forstner, 1994). Mucins, which are heavily O-glycosylated proteins of very high molecular weight, form the most important structural component of mucus (Forstner and Forstner, 1994). A large part of the mucin polypeptide consists of a region highly enriched in serine, threonine, and proline that carries most of the O-glycosylation. This region is composed of tandemly repeated amino acid sequences, which may vary in number between individuals (reviewed in Forstner and Forstner, 1994; Van Klinken et al., 1995). The mucus gel is composed of mucin-oligomers, which can be reduced into their monomeric constituents, resulting in dissolution of the gel (Allen et al., 1982; Strous and Dekker, 1992). Therefore, oligomerization is considered to be essential for gel formation. However, the process of oligomerization is largely unclarified.

Nine different human epithelial mucin genes have thus far been identified and named MUC1–4, 5AC, 5B, 6–8 (Gum et al., 1989, 1990, 1992, 1994; Gendler et al., 1990; Lan et al., 1990; Ligtenberg et al., 1990; Porchet et al., 1991; Bobek et al., 1993; Dufosse et al., 1993; Toribara et al., 1993; Meerzaman et al., 1994; Shankar et al., 1994; Guyonnet-Duperat et al., 1995; Klomp et al., 1995; reviewed in Van Klinken et al., 1995). In addition, human gallbladder mucin (HGBM) and human salivary mucin MG1 were identified in the human gallbladder and salivary glands, respectively (Klomp et al., 1994b; Troxler et al., 1995). Recently, partial sequencing of the cDNA encoding salivary mucin MG1 revealed that this gene is identical to MUC5B (Nielsen et al., 1997). Additionally, we were able to show at the protein level that HGBM is identical to MUC5B (Van Klinken et al., 1996b). Therefore, HGBM and human salivary mucin MG1 represent the gene products of one gene, namely MUC5B. The mucins MUC2, MUC5AC, MUC5B, and MUC7 (Bobek et al., 1993; Klomp et al., 1994a,b; Tytgat et al., 1994) were thus far identified as secretory mucins. Secretory mucins can potentially form mucus gels, whereas for instance MUC1, a membrane-bound mucin, is not capable of forming mucus gels (Ligtenberg et al., 1990). Only partial cDNAs of MUC3, 4, 6, and 8 have been cloned, and cell biological measurements will have to reveal whether these cDNAs code for secretory mucins.

Interestingly, human and rat human MUC2, human MUC5AC, human MUC6, human salivary mucin MG1 (i.e., MUC5B as explained above), X.laevis integumentary mucin-B1, and bovine and porcine submaxillary mucin (Bhargava et al., 1990; Probst et al., 1990; Eckhardt et al., 1991; Gum et al., 1992, 1994; Xu et al., 1992; Meerzaman et al., 1994; Troxler et al., 1995; Toribara et al., 1997) all display homology in their cysteine-rich carboxyl terminal (CT) regions to the cysteine-rich CT regions of human von Willebrand factor (vWF) and Norrie disease protein (NDP) (reviewed in Van Klinken et al., 1995). Disulfide-linked dimerization resides within these CT regions of vWF and NDP (Voorberg et al., 1990, 1991; Meindl et al., 1993; Meitinger et al., 1993). The clinical importance of the CT-region is substantiated by the fact that a mutation in the CT-domain of NDP causes the development of blindness and mental retardation in patients with Norrie disease (Meindl et al., 1993; Meitinger et al., 1993). It is therefore tempting to speculate that oligomerization of the mucins, which display homology to vWF and NDP, proceeds similar to these proteins (Gum et al., 1994).

Very little is known about oligomerization of human gastrointestinal mucins. Human MUC2 precursors have been suggested to form covalently linked dimers in the human colon adenocarcinoma.
cell line, the LS174T (Asken et al., 1995). A family of four mucin genes has been identified on human chromosome 11p15.5 (Pigny et al., 1996). This gene cluster contains four closely adjacent mucin genes (MUC2, MUC5B, MUC5AC, and MUC6) that are expressed in the gastrointestinal tract. These genes are suggested to have evolved from a common ancestor gene, and they show considerable sequence homology. Interestingly, all four mucins contain a very homologous CT-domain. We previously showed that these four mucins were biosynthesized in LS174T cell line (Van Klinken et al., 1996). Cell lines form ideal models to study intracellular processing. Therefore, we performed metabolic labeling studies of the colon carcinoma cell lines LS174T to study the oligomerization of MUC2, MUC5AC, MUC5B, and MUC6. Secondly, we aimed to study the process of oligomerization of MUC2 precursors in more detail, as a representative example, by localizing this process and studying the effect of N- and O-glycosylation on oligomerization.

Results

Precursors of MUC2, MUC5AC, MUC5B, and MUC6 form disulfide-linked oligomers in LS174T cells

As a model to study oligomerization of gastrointestinal mucin precursors, we used the human colon carcinoma cell line, LS174T. To study oligomerization of MUC2, MUC5AC, MUC5B, and MUC6, LS174T cells were pulse labeled with [35S]amino acids for 30 min and chased incubated with unlabeled medium from 0 to 6 h. Mucin precursors were immunoprecipitated and analyzed, without prior reduction, on SDS–PAGE using extended electrophoresis times. These types of analyses have been shown previously to reveal the formation over time of covalent disulfide-bound mucin-precursor oligomers (Dekker and Strous, 1992). MUC2 monomeric precursors were detected at about 600 kDa after 0–6 h chase incubation (Figure 1A, band a). After 1–6 h chase incubation a second band with a lower mobility (Figure 1A, band b) appeared that indicated the formation of oligomeric MUC2 precursors. Monomeric MUC5B precursors were detected at about 470 kDa after 0–6 h chase incubation (Figure 1A, band c); after 4–6 h chase incubation a second band with a lower mobility appeared (Figure 1A, band d) that indicated the formation of oligomeric MUC5B precursors. After 0–1 h chase incubation monomeric MUC5AC precursors were detected as a band of about 500 kDa (Figure 1B, band e), whereas oligomeric MUC5AC precursors were also detected after 0.5–6 h chase incubation at higher molecular masses (Figure 1B, band f). MUC6 monomeric precursors (Figure 1B, band g) were detected at about 400 kDa at 0–1 h chase incubation and MUC6 oligomeric precursors were detected after 0.5–6 h chase incubation at higher molecular masses (Figure 1B, band h). In addition a band was detected on SDS–PAGE, having a slightly lower mobility than the major monomeric MUC6 precursor band g, Figure 1B. This minor band was reproducible in repeated experiments and therefore it could represent an additional biosynthetic form of monomeric MUC6 precursor, rather than a degradative product. Formation of oligomers of MUC2, MUC5AC, and MUC6 precursors all started within 1 h of chase incubation, whereas oligomerization of MUC5B precursors occurred much slower; the first oligomers were detected only after 4 h chase incubation. Precursor bands of each mucin, both mono- and oligomeric, became fainter with chase time, suggesting further processing of these mucins, by, e.g., O-glycosylation. To study the covalent nature of the mucin oligomers, immunopre-cipitated samples were treated with 2-mercaptoethanol, which reduces disulfide bonds. LS174T cells were pulse labeled with [35S]-amino acids for 30 min and chase incubated for 1 h in order to study both mono- and oligomers. After immunoprecipitation with anti-MUC2, MUC2 precursor oligomers were detected on nonreducing SDS–PAGE as a band of high molecular mass (Figure 2, band b), together with some monomeric MUC2 precursors as a band at about 600 kDa (Figure 2, band a). Very little material remained in the slots of the gel, as can be seen as a faint band at the top of the gel (Figures 1A, 2). This material is undefined, but it does not represent multimeric MUC2 nor mature MUC2, as it is detected on both reducing and nonreducing SDS–PAGE. As was previously demonstrated, mature MUC2 enters a 4% reducing SDS–PAGE (Tytgat et al., 1994; Van Klinken et al., 1996a). After reduction the MUC2 oligomers could be reduced into their monomeric constituents, and were found at approximately the 600 kDa position (Figure 2, band a). Very similar results were obtained with other anti-MUC2 antibodies, the monoclonal WE9 and the polyclonal anti-RCM (not shown). Likewise, oligomeric as well as monomeric MUC6 precursors were immunoprecipitated with anti-MUC6 antibody as detected on nonreducing SDS–PAGE (Figure 2, band d). After reduction only monomeric MUC6 precursors were detected (Figure 2, bands c). Oligomeric MUC5AC precursors were detected before reduction (Figure 2, band f), along with monomeric MUC5AC precursors. After reduction only one band, representing monomeric MUC5AC precursor, was detected on SDS–PAGE (Figure 2, band e). As was shown in Figure 1, the oligomerization of MUC5B is relatively slow in LS174T cells. However, in this experiment a small quantity of MUC5B oligomeric precursors (Figure 2, band h) was detected that could be reduced into monomeric precursors (Figure 2, band g). Thus, it can be concluded that all four species of mucin precursors form disulfide bound oligomers in 1 h after pulse labeling.

MUC2 precursors oligomerize in the rough endoplasmic reticulum

Because the oligomerization of the four species of mucin precursors appeared very similar in LS174T, we chose to examine the oligomerization of the different MUC2 precursors in more detail by using inhibitors of cellular processes. Formation of disulfide-bonds is indicated to be a RER-located process (Hwang et al., 1992; Lodish and Kong, 1993). Therefore, covalent oligomerization of mucin precursors most likely takes place early during biosynthesis, namely in the RER. To study whether oligomerization of MUC2 precursors occurs in the RER, we used CCCP and FCCP, which are uncouplers of oxidative phosphorylation and inhibitors of RER-to-Golgi transport (Jamieson and Palade, 1968; Terada, 1981). When LS174T cells were chase incubated for 1 or 4 h in the presence of 0.0, 1 µM of CCCP, similar amounts of monomeric (Figure 3, band a) and oligomeric (Figure 3, band b) MUC2 precursors were immunoprecipitated irrespective of the presence of the inhibitor. Similar results were obtained when FCCP was used (not shown). Therefore, oligomerization was not affected by inhibition of RER-to-Golgi transport. To confirm that CCCP functioned effectively in uncoupling of oxidative phosphorylation, we quantified protein biosynthesis in [35S]methionine/cysteine labeled LS174T cells in the presence or absence of CCCP by autoradiography of SDS–PAGE using a PhosphorImager. After addition of 0.1 or 1 µM CCCP total protein synthesis in LS174T cells was decreased to 25% of the total amount of protein synthesized in the
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Fig. 1. Oligomerization of precursors of MUC2, MUC5B, MUC5AC, and MUC6 analyzed by pulse-labeling and chase incubation. LS174T cells were pulse labeled with [35S]methionine/cysteine for 30 min and chase incubated as indicated (h). Mucin precursors were immunoprecipitated with anti-MUC2, anti-MUC6, anti-MUC5AC, and anti-MUC5B and analyzed under nonreducing conditions (-2ME) on SDS–PAGE. Shown are analyses of MUC2 and MUC5B (A) and of MUC5AC and MUC6 (B). Bands a and b, mono- and oligomeric MUC2 precursors; c and d, mono- and oligomeric MUC5B precursors; e and f, mono- and oligomeric MUC5AC precursors; g and h, mono- and oligomeric MUC6 precursors. The arrowheads indicate the borders between running and stacking gels. The molecular weight markers, unreduced rat gastric mucin dimer (apparent Mr 600,000) and mouse laminin (Mr 900,000) are indicated on the left.

Fig. 2. Analysis of the oligomers of MUC2, MUC5AC, MUC5B, and MUC6 before and after reduction of disulfide bonds. LS174T cells were pulse labeled with [35S]methionine/cysteine for 30 min and chase incubated for 1 h. Mucin precursors were immunoprecipitated with anti-MUC2, anti-MUC6, anti-MUC5AC, or anti-MUC5B and analyzed on SDS–PAGE as for Figure 1 before (-) or after (+) reduction by 2-mercaptoethanol (2ME). Bands a and b, mono- and oligomeric MUC2 precursors, respectively; c and d, mono- and oligomeric MUC6 precursors; e and f, mono- and oligomeric MUC5AC precursors; g and h, mono- and oligomeric MUC5B precursors. Molecular weight markers, unreduced rat gastric mucin dimer (apparent Mr 600,000) and mouse laminin (Mr 900,000) are indicated on the left of each panel.

Absence of CCCP, confirming the efficacy of CCCP in our experiments.

Another approach to localize oligomerization of MUC2 was treatment of LS174T cells with monensin during pulse-chase incubations, which inhibits medial and trans-Golgi transport and functions (Figure 4; Mollenhauer et al., 1990). During pulse chase experiments both MUC2 monomeric (Figure 4A, band a) and oligomeric precursors (Figure 4A, band b) were detected independent of the presence of monensin. All oligomeric MUC2 precursors could be reduced to their monomeric constituents at any time point and at any monensin concentration (Figure 4B). Therefore, MUC2 oligomerization is independent of medial and trans-Golgi functions. Similar results were obtained for the oligomerization of MUC5AC and MUC5B precursors in the
Fig. 4. Effect of monensin on oligomerization of MUC2 precursors. Cells were incubated during 45 min with monensin, pulse labeled with [35S]methionine/cysteine for 30 min and chase incubated in the presence of 0, 0.01, 0.1, or 1 µM monensin throughout the experiment. MUC2 was immunoprecipitated with anti-MUC2 from cell homogenates of monensin treated or untreated cells and analyzed before (-2ME, A) or after reduction (+2ME, B) on SDS–PAGE as for Figure 1. Bands a and b denote monomeric and oligomeric MUC2 precursors, respectively.

presence of monensin (not shown). To confirm that monensin functioned effectively, we quantified mucin sulfation, which is a trans-Golgi localized process, in [35S]sulfate-labeled LS174T cells in the presence or absence of monensin by autoradiography of SDS–PAGE using a PhosphorImager. A marked decrease to 15% in mucin sulfation was noted with concentrations as low as 0.01 µM monensin. This confirmed the efficacy of monensin in inhibiting trans-Golgi functions in our experiments, as reviewed earlier (Mollenhauer et al. 1990).

N-Linked glycans are necessary for efficient oligomerization of MUC2

To study whether N-glycosylation plays a role in oligomerization of MUC2 precursors, we used tunicamycin, an inhibitor of N-glycosylation. After pulse labeling of the untreated control, monomeric MUC2 precursors (Figure 5A, band a) were immunoprecipitated and detected on nonreducing SDS–PAGE. Also some oligomeric MUC2 precursors (Figure 5A, band b) were immunoprecipitated at this time point. When LS174T cells were incubated with different concentrations of tunicamycin, a shift of the monomeric MUC2 precursor band toward a lower molecular mass occurred, due to the absence of N-glycans on these MUC2 precursors (Figure 5A, band a). No oligomeric MUC2 precursors were detected after pulse labeling when tunicamycin was present. After chase incubations of 0.5 and 1 h, oligomeric MUC2 precursors were detected in increasing amounts in the control experiment. Notice that in the samples of the chase incubations of 0.5 and 1 h higher quantities of oligomeric than monomeric MUC2 precursors were detected after the 0.5 and 1 h chase periods. The oligomers that were formed in the presence of tunicamycin have a lower apparent M₄ than monensin. This confirmed the presence of monensin by autoradiography of SDS–PAGE using a PhosphorImager. A marked decrease to 15% in mucin sulfation was noted with concentrations as low as 0.01 µM monensin. This confirmed the efficacy of monensin in inhibiting trans-Golgi functions in our experiments, as reviewed earlier (Mollenhauer et al. 1990).

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Fig. 5. N-linked glycans are necessary for efficient oligomerization of MUC2 precursors. LS174T cells were incubated for 3 h, pulse labeled with [35S]methionine/cysteine for 30 min and chase incubated, in the presence of 0, 5, 10, or 20 µg/ml tunicamycin throughout the experiment. MUC2 was immunoprecipitated with anti-MUC2 from cell homogenates of tunicamycin-treated or untreated cells and analyzed before (-2ME, A) or after reduction (+2ME, B) on SDS–PAGE as for Figure 1. Bands a and b denote MUC2 monomeric and oligomeric precursors, respectively.

O-glycosylation of MUC2 is initialized after oligomerization

Brefeldin A was used to study the relationship between initial O-glycosylation and oligomerization of MUC2. Brefeldin A inhibits RER-to-Golgi transport and induces redistribution of subsets of Golgi-associated proteins, such as glycosyltransferases, to the RER (reviewed in Klausner et al., 1992). For instance, the addition of GalNAc residues to rat gastric mucin precursors occurred in the RER only in the presence of brefeldin A, while normally O-glycosylation occurred only after oligomerization and transport to the Golgi complex (Dekker and Strous, 1990). After 30 min pulse labeling predominantly monomeric and little oligomeric MUC2 precursors were detectable in the absence of brefeldin A (Figure 6A). After addition of 0.1–2 µg/ml brefeldin A, a smear could be detected in the nonreduced pulse labeled samples at a similar location as the oligomeric MUC2 precursors in the control lane that was not treated with brefeldin A (Figure 6A). No smears higher up in the gel were noted in the pulse labeled samples prior to reduction (Figure 6A). Reduction did not alter the mobility of this smear of the MUC2 samples from cells treated with brefeldin A (Figure 6B, band b). Therefore, this smear most likely represents monomeric MUC2 precursor, containing initial O-glycosylation. After chase incubations of 0.5 or 1 h, most MUC2 precursors were oligomeric in the noncontrolled reduction samples of cells that were not treated with brefeldin A (Figure 6A). If MUC2 precursors were immunoprecipitated after 0.5 or 1 h chase incubation from brefeldin A–treated cells, an additional smear was detected in the stacking gel on nonreducing SDS–PAGE (Figure 6A, band c). This smear most likely represents oligomeric MUC2 precursor containing initial O-glycosylation, because after reduction this product was converted into the band denoted as b in figure 6B; the band that very likely corresponds to monomeric MUC2 precursor containing initial O-glycosylation. Therefore, brefeldin A treatment very likely does not interfere with oligomerization and most likely induces initial O-glycosylation of MUC2 precursors. Therefore, this experiment with brefeldin A provides further evidence that oligomerization takes place in the RER, and precedes initial O-glycosylation under normal conditions.

Discussion

We have studied the oligomerization of human gastrointestinal mucin precursors in the human colon carcinoma cell line LS174T. In this cell line, we showed the formation of oligomers of precursors of MUC2, MUC5AC, MUC5B, and MUC6 which could be reduced by 2-mercaptoethanol into their monomeric constituents. Each of these mucin precursors oligomerized into a single species oligomer, with estimated molecular masses between 600 and 1200 kDa. Although these molecular masses are very difficult to measure, it seems that these oligomers possess about twice the molecular mass of their monomeric constituents. Therefore, these oligomeric precursors are most likely dimers.
the oligomerization of these mucin precursors, only homo-oligomers, which are thus likely homo-dimers, and no hetero-oligomers are formed. Because the resolution of the polyacrylamide gel system is low for these very high molecular mass molecules, and in the absence of truly reliable very high molecular mass markers, it is difficult to accurately determine molecular masses of the oligomeric precursors. For these reasons, Sepharose gel filtration is also not a good alternative. We demonstrated earlier that it was impossible to determine the molecular mass of the oligomers of rat gastric mucin precursor using this technique (Dekker et al., 1991).

The four mucins studied in the present study constitute a family of related mucin genes clustered closely on a 400 kb region of chromosome 11p15.5 (Pigny et al., 1996). All these mucins are expressed in the gastrointestinal tract and thought to form mucus-gels. They show sequence conservations in two types of regions that may be relevant to mucin oligomerization: all these mucins contain D-domains and a CT-domain (Gum et al., 1992, 1994; Troxler et al., 1995; Nielsen et al., 1997; Toribara et al., 1997). These domains were initially identified in VWF, a protein that displays similar oligomerization during biosynthesis, and were implicated in the oligomerization process (Voorberg et al., 1990, 1991). In VWF biosynthesis, disulfide-linked dimerization resides within the CT domain and takes place in the RER before multimerization (Voorberg et al., 1990, 1991). MUC2, MUC5AC, human salivary mucin MG1 (MUC5B), MUC6, and porcine submaxillary mucin display striking homologies in their cysteine-rich CT domains to the cysteine-rich CT domain of human VWF and NDP (Gum et al., 1992; Meindl et al., 1993; Metzinger et al., 1993; Toribara et al., 1993; Meerzaman et al., 1994; Troxler et al., 1995; Nielsen et al., 1997; reviewed in Van Klinken et al., 1995). Perez-Vilar and coworkers showed that after transfection of a partial cDNA encoding the cysteine-rich CT region of porcine submaxillary mucin to COS cells, dimerization of this CT-domain occurred (Perez-Vilar et al., 1996). Therefore, in the light of our results we speculate that the capacity for disulfide-linked dimerization of mucin precursors of MUC2, MUC5AC, MUC5B, and MUC6 also resides within their cysteine-rich CT regions. The presence of D-domains in all four mucins further suggests that these domains may also have a function analogous to the function of the D-domains in VWF. Only single, putatively dimeric, precursor oligomers were detected on SDS–PAGE for MUC2, MUC5AC, MUC5B, and MUC6, suggesting that no multimers were synthesized within the time course of our experiments. For clarity, we refer to mucin-multimers as being large complexes of mucin molecules, consisting of many mucin-monomers whereas mucin-oligomers can be di-, tri-, or tetramers. Physicochemical and electron microscopical measurements have demonstrated that isolated secretory mucins are composed of very large multimers (reviewed in Forstner and Forstner, 1994; Van Klinken et al., 1995). Multimerization of dimeric mucin molecules may be a late cellular or even extracellular event, and it cannot be excluded that during this process hetero-multimers are formed between the various mucin species. Because we detected oligomeric mucin precursors early in the biosynthesis, oligomerization must take place before multimerization. Accordingly, Sheehan and coworkers very recently suggested that multimerization of MUC2 in the human colonic cell line PC/AA occurred after glycosylation and dimerization had taken place (Sheehan et al., 1996).

The oligomerization of the precursors of MUC2, MUC5AC, MUC5B, and MUC6 displayed considerable analogy. To study the intracellular processing of these mucins, we chose to examine the biosynthesis of MUC2 precursors in more detail. During incubations of LS174T cells with CCCP and FCCP, inhibitors of RER-to-Golgi transport, or monensin, an inhibitor of medial and trans-Golgi transport and functions, we showed that oligomerization of MUC2 precursors was not affected. Therefore, oligomerization very likely takes place in the RER. Previously, Asker and co-workers suggested that MUC2 dimerizes early in biosynthesis, before O-glycosylation has taken place, by showing that GalNAc residues were not detectable on mono- or oligomeric MUC2 precursors (Asker et al., 1996). We performed incubations with brefeldin A, which also inhibits RER-to-Golgi transport and induces redistribution of Golgi-associated enzymes, such as glycosyltransferases, to the RER (reviewed in Klauser et al., 1992). Previously, it was shown that, after incubations with brefeldin A, rat gastric mucin precursors displayed a high affinity to Dolichos biflorus agglutinin, which binds to GalNAc residues (Dekker and Strous, 1990). Also the galactose incorporation was very low in the latter study, implicating that the initial O-glycosylation of rat gastric mucin precursors was confined to initial α(1–0) GalNAc addition to serine and threonine residues. After brefeldin A incubations in our present study, oligomerization of MUC2 precursors was not inhibited, but smears, instead of distinct bands of monomeric and oligomeric MUC2 precursors, were detected on SDS–PAGE, which most likely results from the addition of α(1–0) GalNAc residues. Therefore, the oligomerization of MUC2 precursors takes place early in biosynthesis, in the RER, and normally precedes O-glycosylation. This is very similar to the findings of rat gastric mucin (Dekker and Strous, 1990). Interestingly, when precocious initial O-glycosylation of MUC2 precursors was induced by brefeldin A, this still allowed oligomerization of the MUC2 precursors. This indicates that oligomerization and initial O-glycosylation are principally independent processes, although these processes are normally physically separated in different compartments. However, definite proof of the presence of O-glycans of brefeldin A–treated MUC2 precursors would have been given by showing affinity for Dolichos biflorus lectin, as was shown for rat gastric mucin (Dekker and Strous, 1990). In the presence of tunicamycin, oligomeric MUC2 precursors did form, but the oligomers displayed lower apparent Mr values on SDS–PAGE due to the absence of N-glycans. Importantly, tunicamycin slowed down, but did not inhibit, the process of oligomerization of MUC2 precursors. By inhibiting the processing of N-glycans with mDNM, no effect was noted on the oligomerization of MUC2 precursors. These experiments also paralleled the findings for rat gastric mucin (Dekker and Strous, 1990). Therefore, oligomerization of MUC2 precursors, although slowed down, can still take place in the absence of N-glycans. Similarly, it was demonstrated that dimerization of transfected CT-domain of porcine submaxillary could also take place in the absence of N-glycans (Perez-Vilar et al., 1996).

After chase incubations in this study no mature mucins were detected on SDS–PAGE. Likely, this is because most analyses were performed under nonreducing conditions, and therefore mature mucins could not enter the stacking or running gel. We previously showed the biosynthesis of mature MUC2, MUC5AC, and MUC5B in LS174T cells (Van Klinken et al., 1996a) and found that formation and secretion of mature mucins was slow, taking up to 20 h for mature mucins to be formed and secreted.

In conclusion, we have shown that the precursors of the chromosome 11p15.5 mucin family, MUC2, MUC5AC, MUC5B, and MUC6, oligomerize, most likely by a common mechanism involving dimerization. As paradigm, the oligo-
Oligomerization of MUC2 occurs in the RER, precedes O-glycosylation, whereas N-glycans are not essential but are necessary for efficient oligomerization of MUC2. Future experiments are necessary to unravel the process of multimerization of these mucin precursors in order to fully comprehend the biosynthesis of the gastrointestinal mucus gel.

Materials and methods

Antibodies

Rabbit polyclonal antisera were used in all experiments, except for one monoclonal antibody WE9, which recognizes a peptide epitope in the unique termini of human MUC2 (Tytgat et al., 1995). Anti-human colonic mucin antisera (anti-HCM, referred to as anti-MUC2 in this study) and anti-rat colonic mucin antisera (anti-RCM), respectively, were raised against purified human and rat colonic mucin and recognize the non-O-glycosylated termini of the polypeptides of human and rat MUC2 (Tytgat et al., 1994, 1995a,b). Anti-rat gastric mucin antisera (anti-RGM, referred to as anti-MUC5AC in this study) was raised against purified rat gastric mucin and recognizes the non-O-glycosylated parts of the polypeptides of both rat and human gastric mucin (Dekker et al., 1989; Klomp et al., 1994a; Tytgat et al., 1995a). By tryptic peptide and cDNA sequencing the human gastric mucin recognized by anti-RGM was identified as MUC5AC (Klomp et al., 1995). Anti-human gallbladder mucin antisera (anti-HGBM, referred to as anti-MUC5B in this study) was raised against purified HGBM and was shown to recognize predominantly the non-O-glycosylated parts of the polypeptide of HGBM (Klomp et al., 1994b; Tytgat et al., 1995a). HGBM was shown to be identical to MUC5B, and therefore anti-HGBM is referred to as anti-MUC5B (Van Klinken et al., 1996b). Anti-MUC6.1, referred to as anti-MUC6, was raised against synthetic peptides representing the tandemly repeated amino acid sequences of MUC6 (De Bolós et al., 1995) and purified by peptide affinity chromatography. The affinity and specificity of anti-MUC6.1 was demonstrated by peptide ELISA (De Bolós et al., 1995). We previously showed that these antibodies were monospecific when used to immunoprecipitate mucin precursors from LS174T cells (Van Klinken et al., 1996a).

Cell culture

LS174T colon carcinoma cell line was obtained from American Type Culture Collection. LS174T cells were cultured in 4 cm² tissue culture wells (Costar, Cambridge, MA). All cells were cultured at 5% CO₂ and 95% relative humidity and 37°C in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Breda, The Netherlands) with 4.5 g/l glucose, 3.7 g/l NaHCO₃, supplemented with 0.1 mM nonessential amino acids (Gibco), 50 U/ml penicillin (Sigma, St. Louis, MO), and 50 µg/ml streptomycin (Sigma). Medium was supplemented with 20% fetal calf serum (Gibco). Medium was replaced daily. Cells were routinely treated with trypsin at near-confluent densities and split 1 to 2. Experiments were performed on LS174T cells just before confluence (passages 116–126).

Metabolic labeling and immunoprecipitation

Metabolic labeling experiments of LS174T cells were performed just before confluence, the time point of high mucin mRNA levels (Van Klinken et al., 1996a). Cell monolayers were washed twice with PBS. Intracellular methionine/cysteine or sulfate was depleted by incubation of 45 min in Eagle’s minimum essential medium (EMEM, Gibco) containing 4.5 g/l glucose, nonessential amino acids, penicillin and streptomycin, but no methionine/cysteine or sulfate, respectively. Cultures were grown at 5% CO₂, 95% O₂ at 37°C and at high relative humidity.

Pulse labeling was performed for 30 or 60 min by adding 35S-labeled amino acids (CellLabeling Mix, Amersham, Bucks., UK; specific activity 1000 Ci/mmol, containing 65% [35S]methionine and 25% [35S]cysteine) to label the polypeptide. Pulse labeling was performed for 60 min with [35S]sulfate (specific activity, 1050 Ci/mmol; Amersham) to label mature sulfated glycoprotein. Pulse labeling was performed on LS174T cells at 20 µCi label per 200 µl medium per well. Chase incubations were performed for up to 6 h by washing the cell monolayer with prewarmed PBS and then adding 1 ml unlabeled complete DMEM per culture well.

After the respective pulse or chase experiments, cell monolayers were washed once with PBS and homogenized at 0°C in buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA, 1% (w/v) Triton X-100 (BDH, Poole, UK), 1 mM phenylmethylsulfonyl fluoride (Sigma), 100 µg/ml pepstatin A (Sigma), 100 µg/ml leupeptin (Sigma), 10 mM iodoacetamide (Sigma), and 0.24 U/ml aprotinin (Sigma). Mucin was immunoprecipitated from the homogenates overnight at 4°C with anti-HCM, WE9, anti-RCM, anti-RGM, anti-HGBM, or anti-MUC6.1 antibodies. For each immunoprecipitation, 20 µl of anti-HCM, anti-RCM, anti-HGBM, or anti-RGM or 50 µl of WE9 or 10 µl of anti-MUC6 were added per ml homogenate. Immunocomplexes were precipitated using Sepharose CL-4B–coupled protein A (Pharmacia, Uppsala, Sweden). Immunoprecipitated mucins were spun down and washed three times with buffer containing 1% (w/v) Triton X-100, 1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (w/v) bovine serum albumin (Boehringer Mannheim, Mannheim, Germany) in PBS, followed by washing twice in PBS diluted 10-fold. Samples were analyzed on SDS–polyacrylamide gel electrophoresis (SDS–PAGE), with or without reduction by 2-mercaptoethanol (2ME, Bio-Rad, Richmond, CA), using a 3% stacking and 4% running gel (Van Klinken et al., 1996a). To ensure that all high molecular weight mucin precursors entered the running gel, electrophoresis was performed for twice the normal time that was necessary for the front to reach the end of the gel. For molecular weight markers, unreduced rat gastric mucin precursors, labeled with [35S]labeled amino acids, were used with apparent molecular masses of about 300 kDa for the monomer and 600 kDa for the dimer (Dekker and Strous, 1990). Prestained high molecular weight markers, with molecular masses between 49.5 and 205 kDa (Bio-Rad), were also used. Gels were fixed in 10% methanol, 10% acetic acid, and incubated for 10 min with Amplify (Amersham). Radiolabeled mucins were analyzed by fluorography, the Biomax MR film (Kodak, Rochester, NY) was exposed for 1 week to 200 µCi label per 200 µl medium per well. Gels were cut out of the film, and the fluorograms were scanned (BioRay, Cambridge, MA) for 3 h, pulse labeled, and chased in the continuous presence of 0, 5, 10, 15, or 20 µg/ml tunicamycin (Calbiochem, Cambridge, MA). Alternatively, LS174T cells were incubated for 45 min with 0, 0.01, 0.1, or 1 µM monensin (The Lily Lab., Indianaplis, IN) or 0, 0.1, 1, or 2 µg/ml brefeldin A (a gift from H. van Baars and J. Stokvis, Sandoz BV, Uden, The Netherlands) or 0, 0.06, 0.6 or 6 mM N-methyl-1-deoxynojirimycin (mDNM, Sigma) followed by pulse labeling and chase incubations in the continuous presence of these inhibitors. Carbonyl cyanide m-chlorophenyl-
hydrazine (CCCP, Sigma) or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, Sigma) were added only during chase incubations of LS174T cells in concentrations of 0, 0.1, or 1 μM.

Quantification
To measure the effectiveness of some inhibitors, LS174T cells were pulse labeled with [35S]sulfate or [35S]methionine/cysteine in the presence of monensin or CCCP, respectively. Total homogenates were analyzed on SDS–PAGE and then incorporation of these labels in cellular proteins was quantified on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

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Abbreviations
CCCP, carbonyl cyanide m-chlorophenylhydrazone; CT domain, carboxyl terminal domain; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; HGBM, human gallbladder mucin; mDNM, N-methyl-1-deoxynojirimycin; 2ME, 2-mercaptoethanol; NDP, Norrie disease protein; PAGE, polyacrylamide gel electrophoresis; RER, rough endoplasmic reticulum; vWF, von Willebrand factor.

References


