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Differential glycosylation of MUC1 in tumors and transfected epithelial and lymphoblastoid cell lines

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The membrane-bound mucin-like protein MUC1 with a specified number of tandem repeats has been expressed by transfection of the cDNAs in both the epithelial cell lines MDCK and LLC-PK$_1$, and human lymphoblastoid cell lines T2 and C1R. The structure and glycosylation states of the MUC1 in these four lines were compared with that of the endogenous MUC1 found in the human pancreatic (HPAF) and breast (BT-20) tumor cell lines using flow cytometry and Western blot analysis with anti-MUC1 antibodies, which are either sensitive or insensitive to the glycosylation state of the tandem repeat, and pretreatment of cells with phenyl-α-galactosaminide, an inhibitor of mucin sialylation. A similar analysis of MUC1 expression in transfected normal and O-glycosylation defective CHO cells reveals that the addition of galactose to the core oligosaccharide structure is apparently responsible for the anomalous difference in M_r between the mature and propeptide forms of the MUC1. Both the tumor cells and the transfected lymphoblastoid cells consistently express significant steady state levels of both the heavily glycosylated mature forms and the poorly glycosylated propeptide forms of the MUC1, whereas MUC1 is found predominantly as the mature extensively glycosylated species in the transfected epithelial cells. Immunofluoresence microscopy of cross sections of the polarized epithelial cells grown on culture filter inserts reveals that the MUC1 is clearly present at the apical surface of the cells, consistent with its expression in normal tissues. Thus, the successful expression of the MUC1 by transfection of either lymphoblastoid cells or epithelial cells yields model systems both for studying the natural structure/function relationships of the protein domains within the MUC1 molecule and for further elucidating the previously reported MHC-independent T-cell recognition of the MUC1.

***Keywords*: MUC1, mucin-like protein, O-linked glycosylation, apical targeting in epithelial cells**

Introduction

The membrane-anchored mucin-like molecule, MUC1, is normally expressed at the apical surface of epithelial cells; but in carcinomas, the cell polarity is lost and MUC1 is expressed at all surfaces of the cell [1]. The MUC1 which is expressed at increased levels on breast tumor cells [1] also exhibits altered glycosylation which contributes to the formation of tumor-specific MUC1 epitopes which are recognized by both a variety of mouse and human antibodies as well as cytotoxic T-lymphocytes [2–6]. Both the presence of less complex O-linked oligosaccharides, due to changes in the activity of the β1,6-glucosaminyl transferase [7], and the likely overall decrease in O-linked glycosylation [8] results in the exposure of the extended peptide backbone [9] which includes a variable number of tandem repeats of 20 amino acids.

Polymorphisms for the number of tandem repeats have been identified in humans and this highly antigenic domain is found in the central portion of the MUC1 [10, 11]. The repetitive sequence in single letter code is *PDTRP*APG*ST*APPAHGV*TS*A and the O-linked glycosylation occurs primarily at the threonine in the ST sequence [8, 12] and secondarily at both the threonine and serine of the TS site [8]. These three residues are positioned near proline residues in a weak consensus which is apparently recognized by the UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferases [13–15]. In contrast, the single T in the sequence *PDTRP* is not glycosylated ([8, 12] and our unpublished data), but both antibody and T-cell recognition of this peptide epitope is greatly affected by the extent of O-glycosylation within the tandem repeat [16, 17]. For example, the BC-3 monoclonal antibody [2] recognizes the peptide epitope regardless of the glycosylation state of the MUC1, while the monoclonal antibodies HMFG-2 [12] and SM-3 [19] recognize either poorly glycosylated or unglycosylated repeats, respectively.

Surrounding this repetitive domain, approximately 100 residues are found in an amino-terminal flanking domain and 328 residues are found at the flanking carboxyl-terminus.

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Both flanking domains contain imperfect copies of the tandem repeats, and at least twenty additional potential sites for O-linked glycosylation are present. The carboxyl-flanking 328 residues make up three distinct domains: (i) a linker domain of 235 residues with five consensus sites for N-linked glycosylation; (ii) a transmembranous domain of 24 residues and (iii) a carboxyl-terminus cytoplasmic domain of 69 residues. Posttranslational cleavage of the MUC1 at a site approximately 65 residues before the membrane anchor (within the linker domain) results in a heterodimeric form of the mature protein with a 20 kDa amphipathic small subunit and a large hydrophilic subunit which includes the repetitive mucin-like sequences [20]. While the subunits remain noncovalently associated at the cell surface, apparently a second *in vivo* cleavage of the larger amino-terminal subunit results in release of the MUC1 amino-terminus with the central mucin-like tandem repeat domain. Prior to this release, it is likely that the MUC1 recycles through the intracellular compartments where at least sialylation of any available sites is continued [21].

The function of the MUC1 on epithelial cells remains unknown. Its extended structure is consistent with an anti-adhesion function which could be important in the development of tubules, while the repetitive mucin-like domain could have a protective function in capturing bacteria [22]. The relatively large cytoplasmic domain may act in signal transduction through interactions with the cytoskeleton [23] although this possibility has not been thoroughly addressed. However, the ability to test the structure/function relationships of these many complex domains is dependent on the development of a faithful model system in which to express recombinant forms of the human MUC1. Although the MUC1 has been expressed on the surface of transfected MDCK cells (dog kidney) and found to inhibit integrin-mediated cell adhesion to extracellular matrix components [24], neither the structure, glycosylation state nor the polarity of expression of the MUC1 was assessed. These issues are addressed in this present study by comparison of human MUC1 forms found in transfected MDCK and LLC-PK_1 epithelial cells with those in human tumor and transfected lymphoblastoid cells, as well as normal and glycosylation-defective Chinese Hamster Ovary (CHO) cells.

The anti-adhesion property of the MUC1 is also thought to play a significant role in the survival and metastasis of tumor cells since its presence could disrupt both normal intercellular interactions [25] and immunosurveillance by cytotoxic lymphocytes [26]. However, a T-lymphocyte MHC-independent recognition of the MUC1 specifically in tumor cells has been reported which relies both on the repetitive nature of the epitopes to cross-link T-cell receptors and the underglycosylation of the molecules [4, 27]. The additional requirements for this type of recognition are under investigation and the studies would be augmented by the availability of model cell lines expressing recombinant forms of the MUC1. The ability of the transfected cell lines described here to serve as targets of specific T-cells is addressed in the companion paper by Magarian-Blander *et al.* [28].

Materials and methods

MUC1 cDNA vector preparation

The pREP4-MUC1-2 and p-REP4-MUC1-22 expression vectors were prepared by ligation of the *Hind*III DNA fragment of either the pJBOF.mucl Δ plasmid [29] containing the MUC1 cDNA with two tandem repeats or the pDKOF.mucl plasmid [16] containing the MUC1 cDNA with 22 tandem repeats, respectively, into the *Hind*III site of the pREP4 plasmid (Invitrogen, USA).

Cell culture and transfection of cells

LLC-PK_1 cells [30] were obtained from K. Amsler (Robert Wood Johnson Medical School, NJ), MDCK II cells [31] were obtained from Drs J. Youngner and P. Whitaker-Dowling (University of Pittsburgh School of Medicine, PA), and CHO and ldlD cells [32] were obtained from M. Krieger (MIT, MA). Cells were maintained at 37 °C, 5% CO_2 in DMEM and HAMS F12 (1:1) media with 3% fetal bovine serum. These four lines were passaged by treatment of cells growing on tissue culture plastic with 0.05% trypsin and 0.53 mM EDTA. All tissue culture reagents were obtained from life Technologies (BRL/GIBCO, USA). MDCK II, LLC-PK_1, CHO and ldlD cells were transfected using 2 μg DNA (pREP4-MUC1) and 6 μg Lipofectamine (BRL/GIBCO) as directed by the manufacturer, and transfected cells were selected by growth in media with 300 μg ml^{-1} hygromycin (Calbiochem, USA). Clonal cell lines were obtained by limiting dilution on multiwell plates. Polarized cultures of either MDCK or LLC-PK_1 cells were obtained by plating cells at confluency (2×10^6 cells) on 25 mm Millicell PCF tissue culture inserts (0.4 μm pores, Millipore, USA) and maintaining the cells for at least 10 days.

Human lymphoblastoid cell lines T2 [33] and CIR [34] were obtained from R. Salter and W. Storkus, respectively (University of Pittsburgh School of Medicine, PA). Pancreatic tumor cell line, HPAF, and breast tumor cell line, BT-20, were obtained from R. Metzger (Duke University, NC) and The American Type Culture Collection, respectively. T2 and CIR cells were transfected by electroporation using a BioRad (USA) Gene Pulser apparatus and 10 μg DNA (pDKOF.mucl or pBOF.muclΔ described above), and transfected cells were selected by growth in media with 0.5 mg m^{-1} Geneticin (G418 from BRL/GIBCO). These cells were maintained at 37 °C, 5% CO_2 in RPMI 1640 with 10% fetal calf serum (Sigma, USA) and passaged by trypsinization. Where noted in the text, phenyl *N*-acetyl-α-D-galactosaminide (phenyl-Gal-Nac, Sigma) was included in the media at 2.0 mg ml^{-1}.

Characterization of MUC1 expression

Flow cytometry

Cells subjected to analysis by flow cytometry were prepared as described previously [3].

Western blot analysis

Cell extracts used for Western blot analysis were obtained directly from confluent cultures of either MDCK II LLC-PK_1, CHO or ldlD cells by removal of the media and incubation of the attached cells in 60 mm dishes for 1 min with 150 µl of cold Hepes-buffered saline (HBS, 10 mM Hepes, pH 7.4, 150 mM NaCl) containing 60 mM n-octyl β-D-glucopyranoside (octyl glycoside, Sigma). The extract was centrifuged for 5 min in an Eppendorf (USA) Microfuge Model 5414 ($\sim 10\,000 \times \mathbf{g}$) at 4 °C to remove insoluble material, and the supernatant was recovered. Where noted in the text, an aliquot of 40 µl was added to 10 µl of Laemmli sample buffer [35], heated for 2 min at 90 °C and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Alternatively, the entire detergent extract (150 µl) was incubated overnight with 50 µl of a 50% slurry of beads conjugated to BC-3 monoclonal antibody prepared from CNBr-activated Sepharose 4B as described by the manufacturer (Sigma). The anti-MUC1 antibody BC-3 was obtained from I. McKenzie (University of Melbourne, Australia). This immuno-complex was subsequently washed with HBS containing 1% Triton X-100 (Sigma) and incubated at room temperature for 35 min with 30 µl 100 mM CAPS buffer, pH 11.5 (Sigma). The 30 µl of eluate was recovered with a Hamilton syringe and heated for 2 min with 20 µl of Laemmli sample buffer prior to SDS-PAGE. Extracts of either T2 or ClR cells (0.25×10^6 cells) were similarly prepared with HBS containing 60 mM octyl glucoside, and heated in Laemmli sample buffer prior to SDS-PAGE. All reagents for SDS-PAGE were obtained form BioRad.

After SDS-PAGE, the proteins were electrophoretically transferred from the gel to Immobilon-NC (Millipore) at 400 mA for 45 min, and the blot was blocked overnight by shaking at 4 °C in phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.5, 150 mM NaCl) containing 5% non-fat dried milk (Carnation Company, USA). The blot was subsequently incubated in PBS with 1% non-fat dried milk and a mouse monoclonal antibody: BC-2 [2] ascites fluid from I. McKenzie (diluted 1:300) at room temperature for 45 min; HMFG-2 [18] or SM-3 [19] hybridoma supernatant from J. Taylor-Papadimitriou (Imperial Cancer Research Fund, UK) (diluted 1:15) overnight a 4 °C. All blots were subsequently washed with PBS and incubated for 35 min with peroxidase-conjugated goat anti-mouse IgG (Sigma). The immunoreactive proteins were visualized using ECL chemiluminescence reagent (Amersham, USA) and X-OMAT AR film (Eastman Kodak Company, USA) as directed by the manufacturer.

Immunofluorescence microscopy

Immunofluorescence microscopy was carried out on polarized cultures of MDCK cells growing on Millicell PCF culture inserts (Millipore). Culture media was removed from the filter inserts, and the cells were washed with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ prior to fixation with 0.5% paraformaldehyde and embedding in Tissue-Tec O.C.T. (American Scientific, USA) at -35 °C. Slices of 5 µm were cut perpendicular to the filter, and placed on superfrost glass slides (Fisher, USA). The slices were incubated with BC-3 mouse monoclonal antibodies against MUC1, and then goat anti-mouse CY3-conjugated antibodies (Jackson Labs, USA).

Results and discussion

Characterization of MUC1 clycosylation in human tumor and transfected lymphoblastoid cell lines

Human breast (BT-20) and pancreas (HPAF) tumor cell lines of epithelial origin were extracted with detergent and subjected to SDS-PAGE and Western blot analysis using the MUC1-specific monoclonal antibody, BC-3, which recognizes the amino acid sequence APDTRP within the tandem repeat regardless of the extent of MUC1 glycosylation. As shown in Fig. 1, two different immunoreactive bands are apparent in each case. Based on the extensive characterization of the structure and synthesis of MUC1 in the ZR-75-1 breast cancer cell line [36] the bands of $M_r > 200\,000$ represent the mature highly glycosylated mucin (M), while the M_r 140 000 bands represent the cleaved propeptide (P) which is poorly glycosylated. The sizes of the bands are consistent with a molecule of MUC1 with ~ 40 tandem repeats although BT-20 cells clearly express a higher ratio of mature to propeptide forms than the HPAF cells. These conclusions are also consistent with the changes in the SDS-gel patterns which result from growth of the tumor cells for 2 days in the presence of phenyl-GalNAc, a competitive inhibitor of oligosaccharide synthesis [37, 38] on the GalNAcα-peptide core of mucins [39]. In each case the M_r 140 kDa form remains unchanged, while the higher M_r form is replaced by an even slower migrating band which corresponds to the poorly sialylated but heavily glycosylated mature form of the MUC1 (M′) previously described by Hilkens and Buijs [36].

The addition of galactose to the mucin-type oligosaccharide cores has been shown previously to produce anomalous behaviour of glycoproteins upon SDS-PAGE. For example, the addition of just 18 galactose residues (FW 180 each, 3240 Da total) to the serine- and threonine-rich domain of the LDL receptor results in an increase in M_r of 30 000 [32] to 40 000 [40]. In an analogous manner, the dramatic increase in M_r for the mature (M) MUC1 when compared to the poorly glycosylated MUC1 propeptide (P) is likely to represent the addition of galactose to GalNAc in the

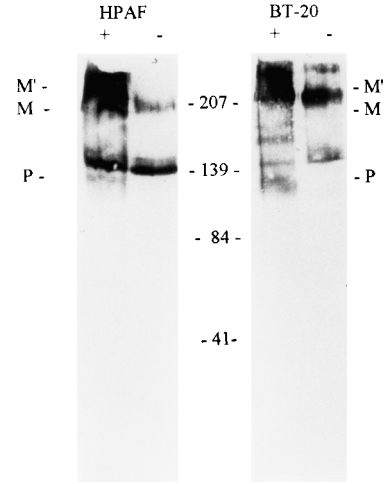

Figure 1. Western blot analysis of MUC1 in tumor cell lines. Extracts of pancreatic (HPAF) and breast (BT-20) tumor cell lines which were grown for 2 days with (+) or without (−) phenyl-GalNAc were subjected to SDS-PAGE and then Western blot analysis using anti-MUC1 monoclonal antibody BC-3. The position of protein molecular weight standards (Sigma) are indicated in the centre as kDa, and bands corresponding to the propeptide (P), mature (M) and undersialylated (M′) MUC1 are indicated on the left and right.

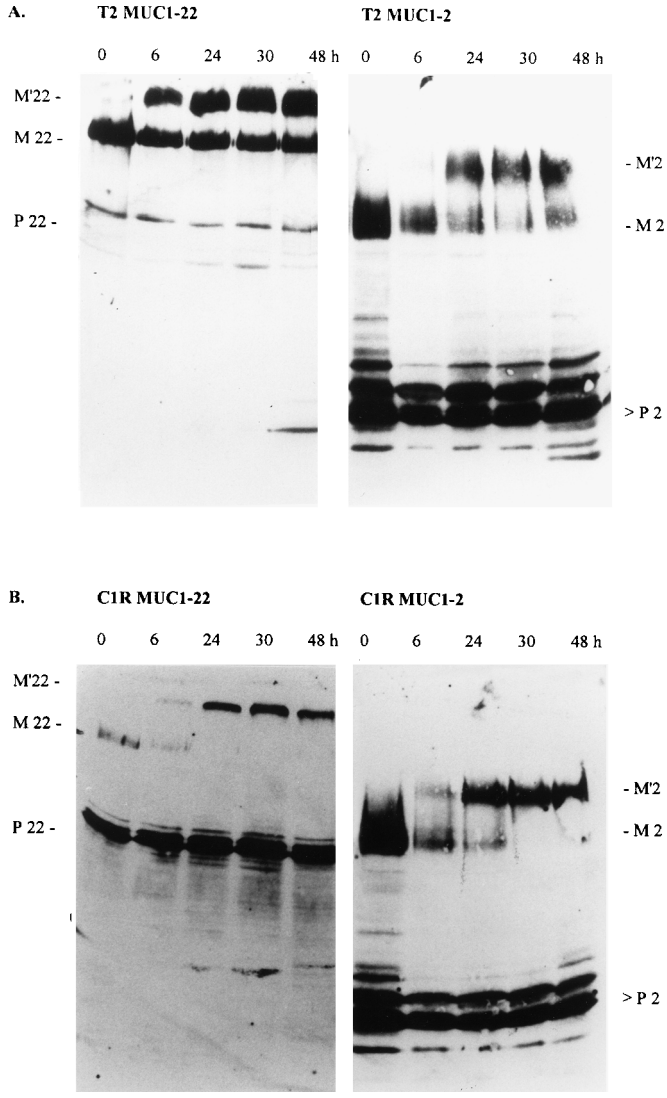

Figure 2. Western blot analysis of MUC1 in transfected lymphoblastoid cell lines. Extracts of transfected T2 (A) or C1R (B) cells expressing either MUC1-2 or MUC1-22 and grown for zero to 48 h ($t = 0$, 6, 24, 30 or 48 h) with phenyl-GalNAc were subjected to SDS-PAGE and Western blot analysis with anti-MUC1 antibody BC-3. The positions for the mature MUC1 (M22 and M2), the undersialylated MUC1 (M′22 and M′2) and the propeptide of MUC1 (P22, P2) for each derivative are indicated on the left and right.

oligosaccharide cores. Since no significant levels of poorly sialylated mature MUC1 are found under normal conditions, the addition of galactose and not sialic acid is apparently the rate limiting step in MUC1 processing. However, the cumulative information would indicate that growth of these human tumor cells with phenyl-GalNAc results in preferential inhibition of sialic acid addition to O-glycans rather than galactose addition to the GalNAc-mucin core. This same conclusion was reached from the characterization of endosialin expression in neuroblastoma cells [41]. Growth of the cells in phenyl-GalNac produced a form of endosialin which was still sensitive to treatment with O-glycanase but had lost its sensitivity to neuraminidase treatment [41].

MUC1 glycosylation was also examined in transfected T2 and C1R cells, described in the companion paper by Magarian-Blander *et al.* [28], which are B-lymphoblastoid for comparison to MUC1 expression in transfected epithelial cells. More specifically, the T2 and C1R cells expressing MUC1 with either 22 or two tandem repeats (Fig. 2) exhibit both the propeptide (P22 and P2) and mature (M22 and M2) forms of the MUC1, respectively. However, the ratio of mature to propeptide for the T2 cells expressing the MUC1-22 (T2MUC1-22) is more like the MUC1 pattern observed for BT-20 cells, while the ratio for the C1RMUC1-22 cells is more like that observed for the HPAF cells. The lower ratio of mature to propeptide forms in these latter cells is likely due to a limited capacity to glycosylate the propeptide since the ratios of mature to propeptide for the MUC1-2 in the T2 and C1R cells are nearly identical. Incubation of the transfected T2 and C1R cells with phenyl-GalNAc over a 2 day period, clearly reveals the accumulation of poorly sialylated mature forms of MUC1 (see Fig. 2, bands M2′ and M22′). The appearance of these less sialylate forms in transfected C1R cells (Fig. 2B) is also coincident with the disappearance of both of the sialylated 200 kDa (M22) and 115 kDa (M2) forms, and corresponds to a $t_{1/2}$ of approximately 6 h. However, the sialylated and under-sialylated forms of the MUC1 are present at similar levels in T2 cells (Fig. 2A) and the tumor cells HPAF and BT-20 (Fig. 1) after growing with phenyl-GalNAc for 48 h, consistent with

a longer half-life for the mature MUC1 in these three cell lines ($t_{1/2} > 24$ h).

Characterization of transfected MUC1 expression in normal and glycosylation-defective CHO cell lines

In order to further characterize the glycosylation pattern of the MUC1 forms, both normal and glycosylation-defective CHO cell lines were transfected with expression vectors encoding the cDNAs for the human CHO cell line, ldlD, lacks the epimerase which produces UDP-Gal or-GalNAc from UDP-Glc or -GlcNAc, respectively. When ldlD cells are grown in low levels of fetal calf serum which severely limits the recovery of Gal and GalNAc through degradation of serum glycoproteins, neither Gal nor GalNAc is added to newly synthesized glycoproteins. For example, when transfected ldlD cells expressing the LDL receptor are grown under these culture conditions [32], only the poorly glycosylated precursor M_r 125 000 is produced. However, addition of both Gal and GalNAc to the media, but not addition of either one alone, results in the synthesis of the 155 000 kDa mature form of the LDL receptor. Since the complete absence of Gal and GalNAc on the LDL receptor [32], but not the absence of the serine- and threonine-rich domain alone [40], produces an unstable LDL receptor, it is clear that even isolated O-linked mucin-type oligosaccharides play a crucial role in protein structure and function.

Transfection of CHO cells with the cDNA for the MUC1 results in the cell surface expression of both the MUC1-2 and MUC1-22 as indicated by the results of flow cytometry (Table 1). However, a very high percentage of cells also reacts with the HMFG-2 and SM-3 antisera indicating that the MUC1 is not extensively O-glycosylated in these cells. This result is consistent with finding the same pattern of reactivity for the MUC1 upon Western blot analysis of the cell extracts using either the BC-3 or HMFG-2 antibodies (see Fig. 3). The presence of evenly spaced bands for the propeptide (P2) and the mature (M22) MUC1 indicates that there is also heterogeneity in the number of N-linked oligosaccharides present in both the MUC1-2 and -22 (see tunicamycin studies in comparison paper by Magarian-Blander *et al.* [28]. However, this finding also emphasizes the fact that the ratios of mature to propeptide forms of the MUC1-2 and -22 are noticably different. This could mean either that the mature MUC1-22 is more stable than the mature MUC1-2 or that the processing of the MUC1-22 propeptide is more efficient than for the MUC1-2 propeptide. However, similar levels of the uncleaved propeptide band are present in both the MUC1-2 and -22 transfectants at M_r 65 000 and 130 000, respectively (Fig. 3a), suggesting that the rates of propeptide cleavage in the two transfectants are similar even if the efficiency of O-glycosylation differs. The clusters of immunoreactive bands near M_r 45 kDa and 65 kDa react with both the HMFG-2 and BC-3 antibodies, which is consistent with the presence of both and cleaved and uncleaved propeptide, respectively, in these cells. The size and even spacing of the propeptide bands are most consistent with the presence of 2, 3, 4 or 5-linked oligosaccharides on the immature MUC-1 molecules.

Table 1. Cell surface expression of MUC1 as determined by flow cytometry. Numbers represent % of gated cells which are positive for reactivity with the specified mouse monoclonal antibodies and FITC-conjugated second antibody.

Cell line[a]	*NO Ab*[b]	*BC-3*	*HMFG-2*	*SM-3*
CHO	4	6	4	5
+ MUC1-2	7	99	73	56
+ MUC1-22	3	99	97	99
ldlD	5	24	7	10
+ MUC1-2	1	57	16	7
+ MUC1-22	4	80	60	37
LLC-PK1	1	4	3	3
+ MUC1-2	4	65	32	4
MDCK II	1	7	2	2
+ MUC1-2	2	98	20	6

[a] Either non-transfected control cells or transfected cells (+MUC1-2 or -22).
[b] Control with no first antibody.

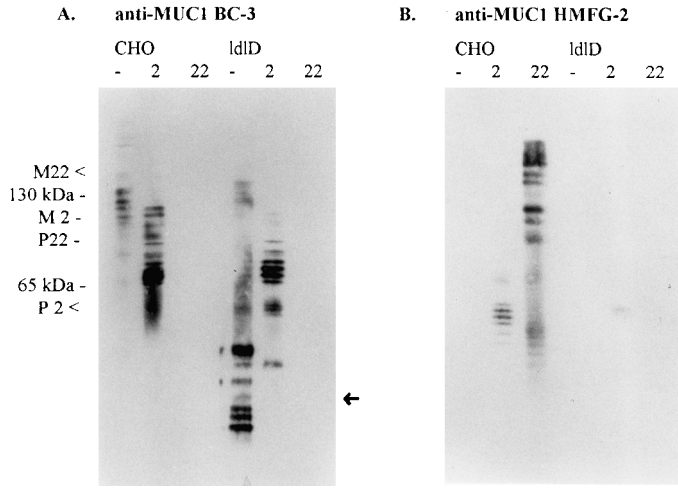

Figure 3. Western blot analysis of MUC1 in transfected CHO cells. Extracts of normal (CHO) and mutant Chinese Hamster Ovary (ldlD) cells transfected either with no cDNA (–) or with the cDNA for MUC1 encoding either two or 22 tandem repeats were subjected to SD-PAGE and Western blot analysis using either anti-MUC1 monoclonal antibodies (A) BC-3 or (B) HMFG-2. The propeptide (P2, P22) and mature (M2, M22) forms of the MUC1 with 2 or 22 tandem repeats, respectively, are indicated on the left. The arrow indicates degradation products within the extracts. See text for details.

Although flow cytometry also reveals surface MUC1 expression for most of the transfected ldlD cells (Table 1), it is clear from Western blot analysis of the cell extracts (Fig. 3) that only the propeptide is produced, and it is minimally glcosylated. As discussed above, these cells would not be expected to carry out mucin-type O-glycosylation due to the lack of Gal and GalNAc, and the lack of mature MUC1-2 or -22 would be consistent with this possibility. Future experiments in which either Gal and/or GalNAc are returned to the culture media should indicate if the dramatic shift of 75 kDa (for MUC1-2) and 90 000 kDa (for MUC1-22) between the propeptide and mature mucin is due to either core (GalNAc addition) and/or peripheral (Gal addition) glycosylation of the peptide core. While sialic acid addition to the LDL receptor results in an increase in M_r of 8000 on SDS-PAGE [40], the addition of sialic acid to MUC1 actually increases the mobility, thereby reducing the M_r by 5000 to 10 000 [36]. It is also clear that this lack of O-linked glycosylation in the ldlD ells produces a MUC1 which is unstable (and/or degraded) within the cell extracts used for SDS-PAGE (see arrow, Fig. 3).

Characterization of the MUC1 expression in transfected epithelial cells

When the epithelial cell lines LLC-PK_1 (porcine kidney) and MDCK (canine kidney) were transfected with the cDNAs for the MUC1-2 and MUC1-22 and selected by growth in hygromycin, only clones expressing the MUC1 with two repeats were obtained. Since clonal lines of CHO and ldlD cells expressing both the MUC1-2 and MUC1-22 have been obtained after transfection with the same vectors, this woud indicate that the problem is not with the pREP4-MUC1-22 vector. However, MUC1 with 22 repeats has been successfully expressed in MDCK cells by transduction with a retroviral vector (unpublished data, R. Henderson, R. Hughey and O. Finn).

In contrast to the MUC1 expression in CHO cells, it is clear from the results of flow cytometry (Table 1) that the majority of the cell surface MUC1-2 in both the transfected LLC-PK_1 (nonclonal) and MDCK (clonal) cells is fully glycosylated. For example, less than 5% of the cells exhibit any reactivity with the MUC1-specific SM-3 monoclonal antibody which would indicate poor glycosylation of a tendem repeat. Consistent with this observation, Western blot analysis of the cell extracts with the BC-3 monoclonal antibody (Fig. 4A) reveals a major band > 100 kDa which in turn shows no reactivity with either the HMFG-2 or SM-3 monoclonal antibodies (data not shown for SM-3). In all cases the propeptide P2 is present as a minor species. However, immunoprecipitation of the MUC1-2 from these epithelial cells with BC-2 monoclonal antibody reveals that differences in glycosylation and processing of the MUC1-2 may exist between

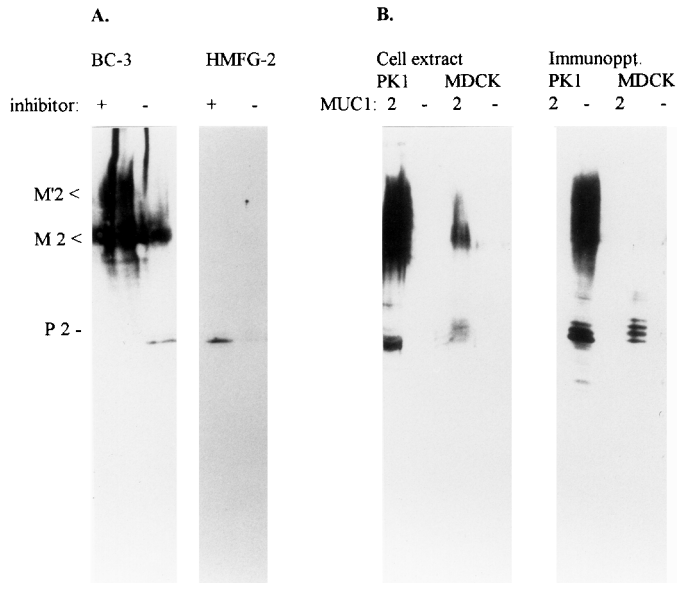

Figure 4. Western blot analysis of MUC1 expression in transfected epithelial cells. (A) Extracts of transfected LLC-PK_1 cells expressing MUC1 with two tandem repeats (MUC1-2) growing for 2 days with (+) or without (−) phenyl-GalNAc (inhibitor) were subjected to SDS-PAGE and Western blot analysis with either anti-MUC1 antibody BC-3 or HMFG-2. (B) Extracts of LLC-PK_1 and MDCK cells (−) or those transfected with the cDNA for the MUC1 with two tandem repeats were either analyzed directly (cell extract) or immunoprecipitated (immunoppt.) with anti-MUC1 antibody BC-2, and subjected to SDS-PAGE and Western blot analysis with anti-MUC1 antibody BC-3. The position of the mature MUC1-2 (M2), the underglycosylated MUC1 (M′2) and the propeptide (P2) are indicated on the far left.

LLC-PK_1 and MDCK cells; the mature MUC1-2 does not appear in the BC-3 Western blot analysis of the BC-2-immunoprecipitated MUC1-2 from the transfected MDCK cells although immunoprecipitation from LLC-PK_1 cells was quite efficient (Fig. 4B).

Lastly, pretreatment of the transfected LLC-PK_1 cells in culture for 2 days with the glycosylation inhibitor, phenyl-GalNAc, produces a slower migrating form of the mature MUC1-2 (M′), which represents the undersialylated form of the mature MUC1 (Fig. 4A). The persistence of the sialylated form of the mature MUC1-2 in these cells after 48 h indicates that the MUC1 is relatively stable in these transfected epithelial cells. Similar results have also been obtained for MUC1 expression in MDCK cells after growth with phenyl-GalNAc (data not shown).

When the transfected MDCK cells are grown on porous filter inserts in culture, a monolayer of polarized cells are formed such that the apical surface is facing the upper media chamber and the basolateral surface is facing the filter and lower media chamber [42]. Immunostaining of cross-sections of the transfected cells growing on these supports, using the anti-MUC1 monoclonal antibody BC-3, indicates that the MUC1-2 is present predominantly at the apical surface of the cells (see Fig. 5). A similar result was obtained

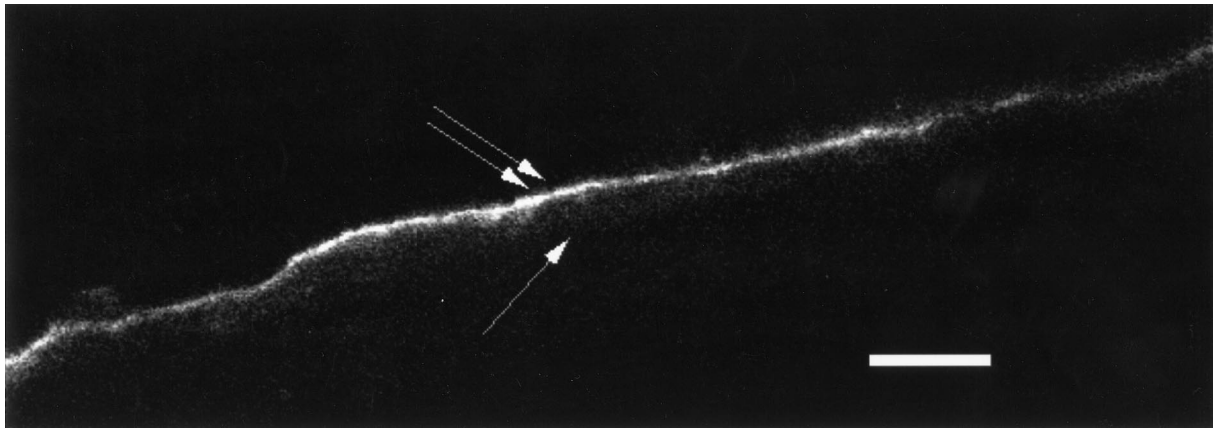

Figure 5. MUC1 is apically expressed in polarized cultures of transfected epithelial cells. MDCK cells were plated at confluency on 30 mm Millicell-PCF culture inserts and maintained for 14 days before preparation of cross-sections as described in Materials and methods. Individual slices were blocked, and incubated with anti-MUC1 antibodies, BC-3, and CY3-tagged goat anti-mouse antisera prior to confocal laser immunofluorescence microscopy. The position of the apical cell surface (double arrows) and basal surface (single arrow) of the cell monolayer are indicated on the photograph. The single bar represents 10 μm.

for transfected LLC-PK_1 cells using scanning confocal immunofluorescence microscopy of the intact filter and analysis of the data in an *x-z* plot (data not shown). This apical localization of the recombinant MUC1 mimics the *in vivo* situation for MUC1 in normal tissue epithelial cells [1]. The cellular mechanism(s) for apical targeting of membrane proteins is not fully understood (for review see [43–46]). While proteins with glycosylphosphatidyl inositol anchors are sorted into apical delivery vesicles in the trans Golgi network by association with patches of glycolipids and cholesterol, the targeting signal(s) for delivery of non-GPI anchored proteins is unknown. In general the anchor-minus forms of these apical transmembranous proteins are still secreted apically, while expression of proteins from non-polarized cells results in non-polarized secretion from these same cells. Thus, the apical targeting signal within the MUC1 molecule is most likely to be within its ectodomian, and the availability of these two model epithelial cell lines will allow further investigation of this possibility.

In conclusion, the expression of MUC1 in transfected T2 and C1R cells produces a pattern of immunoreactive species which is very similar to that observed on tumor cell lines BT-20 and HPAF. Since the observed half-life of the MUC1 in the C1R cells is apparently shorter than MUC1 in these other three cell lines, expression of MUC1 derivatives in C1R cells is likely to represent a better model system for future immunological studies since it can be more readily manipulated. Conversely, MUC1 expression in both LLC-PK_1 and MDCK cells represents ideal model systems in which to further characterize the stable expression of MUC1 in epithelial cells, since it is extensively glycosylated and expressed only at the apical surface of the polarized cells.

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