Oligosaccharides Expressed on MUC1 Produced by Pancreatic and Colon Tumor Cell Lines*

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Michael D. Burdick‡, Ann Harris§, Colm J. Reid§, Takeshi Iwamura¶, and Michael A. Hollingsworth**

From the ‡Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska 68198-6805, §Paediatric Molecular Genetics, Institute of Molecular Medicine, Oxford University, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom, and **First Department of Surgery, Miyazaki Medical College, 5200 Kihara, Kiyotake-cho, Miyazaki 889-16, Japan

MUC1 is expressed at the apical surface of ductal epithelia of tissues, including breast, pancreas, airway, and the gastrointestinal tract, where its functions include lubrication and protection of the epithelia. In addition, roles for MUC1 have been suggested in both adhesive and antiadhesive properties of tumor cells, and extensive O-glycosylation of the MUC1 tandem repeat domain may contribute to these functions. Little information is available on the specific O-glycosylation of MUC1. One problem in identifying different MUC1 glycoforms has been that monoclonal antibodies recognized against the MUC1 core protein recognize epitopes in the tandem repeat domain, which is often glycosylated to an extent that obscures these epitopes. We developed an epitope-tagged form of MUC1 that allowed the detection of multiple MUC1 glycoforms and established the presence of a number of important blood group antibody and tumor-associated carbohydrate antigens on MUC1 expressed by two pancreatic tumor cell lines (Panc-1 and S2-013) and two colon tumor cell lines (Caco-2 and HT-29). Antigens detected include sialyl-Lewisα, sialyl-Lewisβ, sialyl-Lewisγ, and sialyl-Tn.

The human epithelial mucin MUC1 (1–4) is a type 1 membrane-bound glycoprotein expressed by ductal epithelia of a number of organs including breast, pancreas, airway, and gastrointestinal tract (reviewed by Gum (5)). Its functions in these tissues include lubrication and protection of the epithelia, and roles in cell adhesion have been suggested (6–8). There is great diversity in the post-translational processing of MUC1 by epithelial cells in different organs. Moreover, MUC1 is aberrantly expressed by tumors (9) with patterns of post-translational modifications that are different from corresponding normal cell types (10); several different glycoforms of MUC1 were originally described as “tumor-associated antigens.” Although MUC1 has been the subject of a number of lines of research, little is known about the mechanisms that direct its diverse post-translational processing.

Until recently, there has been little precise information available on the O-glycosylation of MUC1 with respect to blood group antigens. One report demonstrated sLeα1 and sLeα epitopes on MUC1 core protein expressed in a pancreatic tumor cell line (11), and two reports showed that MUC1 was one of the proteins associated with SLEX antigen secreted by colon carcinoma cells (12, 13). In addition, the glycosylation of MUC1 purified from a human mammary tumor cell line (T47D), solid tumor, and human breast milk has been described (14).

A major problem in detecting and purifying different glycoforms of the MUC1 core protein is that monoclonal antibodies raised against the core protein bind epitopes in the tandem repeat domain. Many forms of MUC1 that are secreted by different adenocarcinomas are heavily glycosylated in this domain, which obscures the protein epitopes recognized by these anti-MUC1 antibodies. Polyclonal antibodies generated against the cytoplasmic tail (15) do not bind the secreted forms of the protein or some intracellular forms that are proteolytically processed (16). It has been impossible to purify single mucin core proteins by standard biochemical approaches because the biophysical properties of most mucin core proteins are very similar. We developed an epitope-tagged form of MUC1 to circumvent these problems and facilitate analysis of its post-translational processing. The use of an antibody to the epitope tag allows the detection of multiple MUC1 glycoforms, even with a background of endogenous MUC1 and other mucin-like core proteins. In this report, we identify carbohydrate epitopes that are found on MUC1 expressed in two pancreatic tumor cell lines and two colon tumor cell lines.

EXPERIMENTAL PROCEDURES

Reagents—Restriction enzymes and ligase were purchased from New England Biolabs. PNGase F was purchased from Boehringer Mannheim. Oligonucleotides were synthesized by the Eppley Institute Core Laboratory. Monoclonal antibodies were obtained from the following sources: mAb M2 was purchased from IBI; CO514, CO431, B93.1, and CSLEX1 were the gift of Mark Reddish of Biomera Corp.; CA19–9 was a gift from Hilary Koprowski; HMPG-2 was the gift of Sandra Gendler; CA-2 was the gift of Michael Bramwell; and CC49 was the gift of David Colcher. FITC-conjugated goat anti-mouse IgG was purchased from Life Technologies, Inc., peroxidase-conjugated goat anti-mouse Ig was purchased from Western Biotechnology Associates, and peroxidase-conjugated rabbit anti-mouse IgG serum was purchased from Sigma. Enhanced chemiluminescence kits were purchased from Amersham Life Sciences and Pierce. Lipofectin was purchased from Life Technologies, Inc. Cell culture medium was purchased from Célox or Life Technologies, Inc., and fetal bovine serum and Genetecin (G418) were

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1 The abbreviations used are: Leα, Lewisα; Leβ, Lewisβ; Leγ, Lewisγ; sLeα, sialyl-Lewisα; sLeβ, sialyl-Lewisβ; sLeγ, sialyl-Lewisγ; sTn, sialyl-Tn; PNGase F, peptide-N-glycosidase F; ECL, enhanced chemiluminescence; MEM, minimal essential medium; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline.

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Introduction of the Epitope Tag—A double-stranded synthetic oligonucleotide was designed to encode the amino acid sequence DYKDDDD-DYKDDDD in which DYKDDDD is the Flag epitope recognized by the monoclonal antibody M2 (17). The remainder of the inserted amino acid sequence (QILDMVA) is a result of the addition of two unique restriction enzyme sites, BglII and NcoI, which were included to facilitate screening for successful insertion of the double-stranded oligonucleotide and for future cloning purposes. This oligonucleotide was ligated into a BamHI site at position 232 of the MUC1 cDNA (Fig. 1B). In-frame insertion of the oligonucleotide was verified by sequence analysis.

Cell Culture—Panc-1 and S2-013 cells were cultured in MEM supplemented with 5% fetal bovine serum. Caco-2 and HT-29 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Caco-2 and HT-29 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Caco-2 and HT-29 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Caco-2 and HT-29 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum.

Preparation of Cell Lysates—Cells were cultured on coverslips to 80% confluence, stained using mAb M2 (10 μg/ml) and a goat anti-mouse Ig-FITC-conjugated. After incubation in secondary antibody, the cells were washed three times with cold PBS and fixed on ice for 15 min in 4% formalin. The cells were then washed once in PBS, and imaged on either a Zeiss inverted microscope with epifluorescence optics or a Zeiss LSM 410 confocal laser scanning microscope.

FACS Analysis—Adherent cells were released from culture flasks by treating with 0.05 mM trypsin and 1.5 mM EDTA in PBS for 4 min at 37 °C. All subsequent steps were carried out on ice. Cell surface-associated MUC1F is stable to this treatment. The cells were resuspended in 5% MEM and incubated with the anti-epitope tag antibody at a concentration of 10 μg/ml for 30 min. The cells were washed again, resuspended in 5% MEM, and analyzed on a FACStarPLUS (Becton Dickinson).

PNGase F Treatment—MUC1F was immunopurified as outlined above. Immunopurified material was incubated overnight with 1.2 units of PNGase F supplemented with 10 μl of EDTA at 37 °C. The total reaction volume (50 μl) was loaded in a single well for electrophoresis as described above for immunoblotting.

RESULTS

Introduction of the Flag Epitope Tag—A schematic of the MUC1 cDNA construct used in this study is shown in Fig. 1A. The DNA encoding the Flag epitope was added to the MUC1 cDNA (Fig. 1A) by ligating a double-stranded oligonucleotide into a BamHI site found 5' to the region of the cDNA that encodes the tandem repeat domain. This resulted in the in-frame insertion of the sequence DYKDDDDQILDMVA in the amino-terminal side of the tandem repeat domain in the translated MUC1 core protein sequence (Fig. 1B, MUC1F). A site toward the amino terminus of the MUC1 protein was chosen for the insertion because this yields recombinant product with the epitope tag on the extracellular surface of transfected cells, which allowed for convenient screening using surface immunofluorescence microscopy or fluorescence-activated cell sorting. Furthermore, it

Immuno blotting—Cell lysates and immunoprecipitated protein were resolved on 6% denaturing polyacrylamide gels (3% polyacrylamide stacking gel), transferred to polyvinylidene difluoride membranes electrophoretically, and blocked in 5% dry milk in TBS (0.9% NaCl, 10 mM Tris, pH 7.4, 0.5% MgCl2) overnight. Primary antibodies were diluted 1:2000 in blotto (5% nonfat dry milk in TBS). Incubations were for 1 h at room temperature and were followed by three 10-min washes with blotto. Horseradish peroxidase-conjugated secondary antibodies were diluted 1:2500 in blotto, and incubations were for 1 h at room temperature. Second antibody incubations were followed by three washes as above. ECL reagents were applied as per the manufacturer's instructions, and the blots were exposed to ECL-sensitive film (Amer sham Life Sciences).

Immunofluorescence Microscopy—Cells were cultured on coverslips and allowed to grow to approximately 60% confluence. After washing once with PBS, the cells were chilled on a bed of ice for 10 min, then incubated on ice in cold 5% MEM with 10 μg/ml M2 antibody for 1 h. After incubation with primary antibody, the cells were washed three times with ice-cold 5% MEM. The cells were then incubated on ice in cold 5% MEM with 10 μg/ml secondary antibody (goat anti-mouse Ig-FITC-conjugated). After incubation in secondary antibody, the cells were washed three times with cold PBS and fixed on ice for 15 min in 4% formalin. The cells were then washed once in PBS, and imaged on either a Zeiss inverted microscope with epifluorescence optics or a Zeiss LSM 410 confocal laser scanning microscope.

The results are shown in Figs. 2A and 2B. The cell lines were grown to approximately 60% confluence, washed twice in serum-free MEM, and then incubated 12 h in 1 ml of growth medium containing G418 at 500 μg/ml. After incubation with primary antibody, the cells were washed three times with cold PBS and fixed on ice for 15 min in 4% formalin. The cells were then washed once in PBS, and imaged on either a Zeiss inverted microscope with epifluorescence optics or a Zeiss LSM 410 confocal laser scanning microscope.
was predicted that placing the epitope tag at this position in the molecule would be unlikely to interfere with basic biological functions of epitope-tagged protein because the amino acid sequence of this region is poorly conserved among different species (19–21).

Transfection of Cell Lines and Expression of MUC1F—Four cell lines were chosen in which to evaluate the epitope-tagged MUC1. The first, Panc-1, is a poorly differentiated human pancreatic adenocarcinoma cell line that expresses low levels of endogenous MUC1 (and other mucin-like glycoproteins) and relatively low levels of other O-glycosylated glycoproteins (22). The second, S2-013, is a human pancreatic tumor cell line that is well differentiated and known to extensively O-glycosylate mucin-like proteins (23). Caco-2 and HT-29 are human colon tumor cell lines of mixed epithelial morphologies that express other mucin core proteins and tumor-associated carbohydrate epitopes (24–26). The MUC1F cDNA was subcloned at the BamHI site of the expression vector pH8-APr1-neo vector, which we have previously used for establishing stable expression of MUC1 in a number of human and rodent cell lines (27, 28), and transfected into the four tumor cell lines.

Analysis of the primary sequence of MUC1 reveals the presence of five consensus N-linked glycosylation sites, at least some of which are known to be utilized (29). To confirm N-glycosylation of MUC1F and that insertion of the epitope tag near the signal sequence did not interfere with insertion of MUC1F into the endoplasmic reticulum, MUC1F expressed by Panc-1 was immunoprecipitated and treated with PNGase F. PNGase F treatment of MUC1F resulted in an increase in mobility on denaturing SDS-polyacrylamide gel electrophoresis (Fig. 2), demonstrating N-glycosylation of MUC1F.

To test whether insertion of the epitope tag interfered with surface expression of MUC1F, a polyclonal population of Panc-1 cells expressing MUC1F was evaluated for surface expression of the Flag epitope by immunofluorescence. Approximately 25% of the cells surviving G418 selection were positive for expression of the Flag epitope in this assay (data not shown). Clonal populations of transfected Panc-1 cells displayed cell surface expression of MUC1F as detected by confocal microscopy and flow cytometry. Similar cell surface expression was seen in all transfected cell lines. Analysis by confocal microscopy of unpermeabilized Panc-1 cells expressing MUC1F showed a rim fluorescence pattern indicative of surface localization of the MUC1F protein (Fig. 3E). Untransfected Panc-1 cells were unreactive with the M2 antibody (Fig. 3B). The results of flow cytometry experiments showed that greater than 98% of unpermeabilized Panc-1 cells expressing MUC1F were positive for surface expression of MUC1F (Fig. 3F).

Molecular Analysis of MUC1 O-Glycosylation—Immunoprecipitated MUC1F protein derived from lysates of individual clones of the four cell lines stably expressing MUC1F was analyzed for the presence of O-linked carbohydrate structures by Western blotting with a panel of antibodies against a number of tumor-associated and blood-group carbohydrate antigens (Fig. 4, Table I). For all cell lines except Panc-1, multiple clones were evaluated. In each experiment, the anti-epitope tag antibody M2 was used to immunoprecipitate recombinant MUC1F glycoforms. The immunoprecipitated MUC1F was analyzed for expression of blood group antigens by Western blotting. Representative examples of Western blot data for the four cell lines is shown in Fig. 4.

The profile of reactivities of the antibodies with MUC1F was different for each of the four cell lines tested (Table I). Of the nine antibodies evaluated in this study, only CC49 (anti-sTn) was reactive with MUC1F expressed by Panc-1, while antibodies to Leα, sLeα, Leb, sLeb, Leβ, sLeβ, and Leγ structures were by Western blotting with a panel of antibodies against a number of tumor-associated and blood-group carbohydrate antigens (Fig. 4, Table I). For all cell lines except Panc-1, multiple clones were evaluated. In each experiment, the anti-epitope tag antibody M2 was used to immunoprecipitate recombinant MUC1F glycoforms. The immunoprecipitated MUC1F was analyzed for expression of blood group antigens by Western blotting. Representative examples of Western blot data for the four cell lines is shown in Fig. 4.

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Glycosylation of Epitope-tagged MUC1

Pancreatic and colon tumor cells produce and secrete mucin-like molecules that contain complex oligosaccharide structures that correspond to several important blood group and tumor-associated antigens, such as those recognized by the monoclonal antibodies DUPAN-2 (sLe\(^a\)), CC49 (sTn), and CA19-9 (sLe\(^x\)). However, not every O-glycosylated protein that is expressed by pancreatic adenocarcinoma cells receives sLe\(^a\), sTn, or sLe\(^x\) structures as part of their O-linked oligosaccharides, and in many cases it is not known which mucin-like core protein (or proteins) carries a given carbohydrate structure. We expressed an epitope-tagged MUC1 isoform (MUC1F) in four tumor cell lines and evaluated this epitope-tagged protein for the presence of nine blood group and tumor-associated antigens.

Although there were some similarities in the types of oligosaccharides that were added to MUC1F by the different cell lines, each of the four tumor cell lines glycosylated MUC1F with a distinct set of carbohydrate structures. One simple structure, sTn, was found on MUC1F produced by all four tumor cell lines. Consistent with previous reports, a cell line derived from a pancreatic adenocarcinoma (S2-013) expressed the DUPAN-2 epitope (sLe\(^a\)), but the cell lines derived from colon carcinomas did not. It was also notable that sLe\(^a\) was found on MUC1F expressed by S2-013 cells and both of the colon tumor cell lines.

For all cell lines evaluated here, carbohydrate epitopes not detected in untransfected or control transfected cell lysates were found on MUC1F. For example, MUC1F expression in S2-013 cells results in its glycosylation with sLe\(^a\) structures that could be bound by the antibody DUPAN-2. This result, coupled with the observation that DUPAN-2 does not bind to proteins in control S2-013 extracts, suggests that the appearance of this epitope is correlated with expression of the MUC1 protein. It is possible that expression of MUC1F provides a proper protein scaffold for the creation of epitopes that are detectable by these antibodies. For some of the antibodies used in this study, patches of carbohydrate moieties in a particular array may be required for antibody binding and this may be provided by the MUC1 tandem repeat. This leaves open the possibility that similar oligosaccharide structures exist in the cell on endogenous O-glycosylated proteins or glycolipids, but they are not detected by Western blotting because the array provided by these protein backbones may not bind efficiently to these antibodies.

Another hypothesis that would explain the appearance of these structures after transfection with the MUC1F cDNA is that the primary amino acid sequence of MUC1F contributes to its glycosylation. For example, sequence motifs in the MUC1 protein may cause this protein to pass through the Golgi in a way that facilitates the construction of particular oligosaccharide structures by using glycosyltransferases that are constitutively expressed in a given cell type. This does not imply that MUC1 is the only protein that will receive a sLe\(^a\) structure in the S2-013 cells for example, only that it may contain sequence information that helps determine that this carbohydrate structure is created on this molecule by this cell line.

The appearance of sTn oligosaccharide structures after transfection of the MUC1F cDNA may be a result of high levels of expression of MUC1F protein. Overexpression of a protein with multiple acceptor sites for O-glycosylation may saturate the post-translational modification system such that novel, truncated structures are created. This may explain in part the appearance of short oligosaccharides such as sTn on MUC1F in the studies reported here, and on MUC1 and other mucins that are overexpressed by tumors.

There are reports that MUC1 is phosphorylated on tyrosine residues in the cytoplasmic domain and that MUC1 associates with Grb2 and the Sos/Ras exchange protein (30, 31). Although

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**TABLE I**

Summary of Western blot analysis of MUC1 glycosylation by cell lines

| Antibody | Structure | Cell line  | Panc-1 | S2-013 | Caco-2 | HT-29 |
|----------|-----------|------------|--------|--------|--------|--------|
| CO514    | Le\(^a\)  |            |        |        |        |        |
| B67.4    | sLe\(^a\) | +/+        |        |        |        |        |
| CA19-9   | sLe\(^x\) |           |        |        |        |        |
| CO431    | Le\(^b\)  |            |        |        |        |        |
| DUPAN-2  | sLe\(^a\)|            |        |        |        |        |
| B93.1    | Le\(^a\)  |            |        |        |        |        |
| CSLEX1   | sLe\(^x\) |            |        |        |        |        |
| B32.21   | Le\(^b\)  |            |        |        |        |        |
| CC49     | sTn       |            |        |        |        |        |

Where two clones were evaluated, the results for each clone is reported. For example, “+/−” would indicate that clone 1 was negative, and clone 2 was positive.

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**FIG. 4.** MUC1-associated carbohydrate epitopes detected by monoclonal antibodies. Examples of Western blot data from Panc-1, S2-013, Caco-2, and HT-29 cell lines stably expressing MUC1F. mAb M2 was used to immunoprecipitate the epitope-tagged MUC1. mAbs CA19-9, DUPAN-2, CC49, CSLEX1, and CO514 bind carbohydrate epitopes sLe\(^a\), sLe\(^x\), sTn, sLe\(^a\), and Le\(^a\), respectively. Lanes labeled C are loaded with control immunoprecipitated material. Lanes labeled T, T1, and T2 are loaded with immunoprecipitated material from clonal cell lines stably expressing MUC1F. One MUC1F-transfected Panc-1 clone was evaluated, and two clones of transfected S2-013, Caco-2, and HT-29 cell lines were evaluated.
there is no published evidence supporting this hypothesis, it is possible that overexpression of MUC1F in these cells may influence signal transduction events that affect the types or levels of expression of certain glycosyltransferases that are required to create oligosaccharide structures.

It is significant that carbohydrate structures such as sLe\textsuperscript{a} and sLe\textsuperscript{a} are attached to MUC1 by tumor cells. MUC1 expression has been associated with a reduction in ability of cells growing in culture to aggregate (8). This property of MUC1 is thought to result from its rigid and extended structure at the surface of the cell combined with an overall negative charge as a result of extensive sialylation. This may cause MUC1 to physically interfere with cell adhesion molecules by masking their presence on the surface. Blood group antigens on MUC1 may also contribute to adhesion properties of tumor cells. It may be advantageous for tumor cells to express an array of blood group antigen structures on cell surface-associated mucins such as MUC1 because this would allow them to mimic host mechanisms for leukocyte trafficking during the metastatic process. The recently cloned physiologic P-selectin ligand PSGL-1 (32) has a structure that is similar to MUC1 (with tandem repeats, a transmembrane domain, and cytoplasmic tail) and receives O-glycosylation with sLe\textsuperscript{a} and sLe\textsuperscript{a} structures. These carbohydrate structures are known to bind to P-selectin expressed on activated endothelia and this interaction is an early event in leukocyte recruitment and extravasation at sites of inflammation (reviewed by Varki (33)). Tumor cells expressing MUC1 and carbohydrate structures such as sLe\textsuperscript{a} and sLe\textsuperscript{a} may utilize these in cell-cell interactions during the metastatic process. Alternatively, secreted soluble forms of MUC1 carrying oligosaccharide blood group antigens may compete with selectin ligands on leukocytes and interfere with leukocyte trafficking to the tumor. This may explain in part how tumors evade infiltration and recognition by the immune system.

In summary, the generation of an epitope-tagged version of MUC1 allowed us to identify O-linked oligosaccharide structures including Lewis blood group and tumor-associated antigens found on MUC1 expressed by pancreatic and colon tumor cell lines. These studies lay the groundwork for further examination of the relationship between the primary structure of MUC1 and related O-glycosylated proteins, their post-translational processing in different cell types, and their biological functions.

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