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Diverse Glycosylation of MUC1 and MUC2: Potential Significance in Tumor Immunity[1]

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Mucins are major epithelial luminal surface proteins and function as a physical and biological barrier protecting mucous epithelia. Diverse glycosylation of mucins potentially provides a basis for tissue-specific interaction with the milieu. When mucins are associated with malignant epithelial cells, they not only protect these cells from a host environment during metastatic dissemination but also generate immunogenic epitopes which are used by the host in the detection and immunological elimination of carcinoma cells potentially depending upon their status of glycosylation. Diverse mucin structures are generated by the combination of different core peptides, of which 10 have been reported so far, multiple types of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (pp-GalNAc-Ts), and the consequences of stepwise glycosylation events. For example, the mucin 1 (MUC1) associated with malignant cells was previously believed to exhibit unique features with a lower percentage of threonine and serine residues attached to *N*-acetylgalactosamine and/or without extension through core 2 structures. Some of MUC1-specific monoclonal antibodies and cytotoxic lymphocytes recognize the peptide sequences PDTR within the tandem repeat portion exposed by decreased degree of glycosylation. The specific arrangement of *N*-acetylgalactosamine residues is shown to be generated by a combination of pp-GalNAc-Ts with different specificities. The role of core 2 branching is somewhat confusing because well-known carcinoma-associated carbohydrate epitopes such as sialyl-Le^x, sialyl-Le^a, Le^y, and others are often expressed when *O*-glycans are extended through core 2 branching. The other series of well-known carcinoma-associated carbohydrate structures are truncated *O*-glycans, conventionally called Tn and sialyl-Tn antigens. Interestingly, these are often found to be aligned on core polypeptides, resulting in three or more consecutive truncated *O*-glycans. MUC2 and other mucins, but not MUC1, have unique tandem repeats containing three or more consecutive serine or threonine residues, which potentially serve as a scaffold for trimeric Tn and sialyl-Tn epitopes. We recently found, using the MUC2 tandem repeat, that trimeric Tn is a high-affinity receptor for a calcium-type lectin expressed on the surface of histiocytic macrophages. The biosynthesis of trimeric Tn was strictly regulated by the acceptor specificity of pp-GalNAc-Ts. These results strongly suggest that variation in both glycan structures and distribution of glycans on the core polypeptides give mucins unique and diverse biological functions that play essential roles in carcinoma-host and other cellular interactions.

Key words: UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases, cancer, *O*-glycosylation, immunity, mucin.

1. Introduction: mucins and their association with malignant epithelia

1.1. Background. Mucins, the major epithelial luminal surface glycoproteins, are characterized by their high molecular masses (>200 kDa) and high content of carbohydrate side chains (~90%). In humans, at least 10 distinct epithelial mucin core polypeptide genes have been identified. They are designated as *MUC1* (*1-4*), *MUC2* (*5*), *MUC3* (*6*), *MUC4* (*7*), *MUC5AC* (*8*), *MUC5B* (*9*), *MUC6* (*10*), *MUC7* (*11*), *MUC8* (*12*), and *MUC9* (*13*), numbered according to the order of the gene discovery. They share a common feature of a tandem repeat domain rich in serine and threonine residues (Table I). Most of the carbohydrate chains are linked through *N*-acetylgalactosamine residues

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Abbreviations: CRD, carbohydrate recognition domain; CTL, cytotoxic lymphocyte; hMGL, human macrophage calcium-type lectin; mAb, monoclonal antibody; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; pp-GalNAc-T, UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases.

forming so-called *O*-glycans. Theoretically, mucins are the most polymorphic of all biological macromolecules produced by eucaryotic organisms, even more so than immunoglobulin and T cell receptors. They contain several hundred *O*-glycosylation sites (Ser and Thr) GalNAc residues may attach and then be further extended and modified at their peripheral portion in a unique manner (Table II).

1.2. Nomenclature. Although *MUC1-9* theoretically represent the names of gene loci, the same designations are also widely used to describe mucin molecules. However, a splicing variant of the *MUC1* gene product lacking the mucin domain has been reported (*14*). Therefore, strictly speaking only the *MUC1* gene product with *O*-glycans should be called MUC1 mucin. Mice and other mammals have a *muc1* gene, the product of which is considered to be the counterpart of human MUC1. The C-terminal cytoplasmic domain of this molecule was reported to be highly homologous to that of human MUC1 mucin (*15*). Although mouse muc1 has a highly glycosylated tandem repeat extracellular domain, its nucleotide and amino acid sequences did not seem to correspond to those of human MUC1 (*16*).

Mucin *O*-glycans are structurally diverse. Some of them serve as epitopes representing blood group and related antigens with genetic polymorphism. The structural variations of the pattern of extension of *O*-glycans are classified according to the type of monosaccharides attached to the innermost GalNAc and the position of the substitution. They are often termed core 1-4 (Fig. 1) (*17*).

1.3. Carcinoma-associated mucins. Mucins, particularly MUC1, have been a strong focus of attention for many tumor immunologists because they were originally shown to exhibit a breast carcinoma-specific epitope (*18*). Both antibody and T lymphocyte responses to MUC1 were found in a variety of cancer patients (*19-22*). Their immune responses were demonstrated to be specific for the PDTR sequence within the tandem repeat of MUC1. Furthermore, MUC1 has been used as an immunotherapeutic target to elicit both humoral and cellular immunity. MUC1 on cell surfaces was also reported to function as an anti-adhesive molecule, which enhanced malignant tumor cell dissemination (*23*). Such paradoxical functions of MUC1 in the host interactions with carcinoma cells are at least in part due to its heterogeneous glycosylation as discussed below. Some of the precursor structures of *O*-glycans, such as Galβ1-3GalNAc-Thr/Ser (T: Thomsen-Friedenreich antigen), GalNAc-Thr/Ser (Tn antigen), and Sialylα2-6GalNAc-Thr/Ser (sialyl-Tn antigen), are also known as epitopes expressed on epithelial cells under pathological conditions, particularly in association with carcinomas (*24*). MUC2 is one of the scaffold of such epitopes often expressed in a consecutive manner. Although the roles of these truncated *O*-glycans in malignant behavior are not well-understood, they are immunogenic and useful as a vaccine. Their increased expression also corresponds often to advanced and aggressive phenotypes (*25*). This short review is intended to introduce new findings made in our and other laboratories on the regulation of glycosylation of MUC1 and MUC2.

TABLE I. **Tandem repeats of epithelial mucins.** Each sequence shown in the table represents a typical example. In most cases, there are variations in the sequence from one repeat to another. MUC5B has a super repeat structure. The range of genetic variation is not fully understood.

Mucin	Typical amino acid sequence (number of residues)
MUC1	PGSTAPPAHGVTSAPDTRPA (20)
MUC2	PTTTPITTTTTVTPTPTPTGTQT (23)
MUC3	HSTPSFTSSITTTETTS (17)
MUC4	TSSASTGHATPLPVTD (16)
MUC5AC	TTSTTSAP (8)
MUC5B	SSTPGTAHTLTVLTTTATTPTATGSTATP (29)
MUC6	SPFSSTGPMTATSFQTTTTYPTPSHPQTTLPTHVP-PFSTSLVTPSTGTVITPTHAQMATSASIHSTPTGT-IPPPTTLKATGSTHTAPPMTPTTSGTSQAHSSFST-AKTSTSLHSHTSSTHHPEVTPTSTTTITPNPTSTG-TSTPVAHTTSATSSRLPTPFTTHSPPTGS (169)
MUC7	TTAAPPTPSATTPAPPSSSAPPE (23)
MUC8	TSCPRPLQEGTPGS (14)
MUC9	GAMTMTSVGHQSMTP (15)

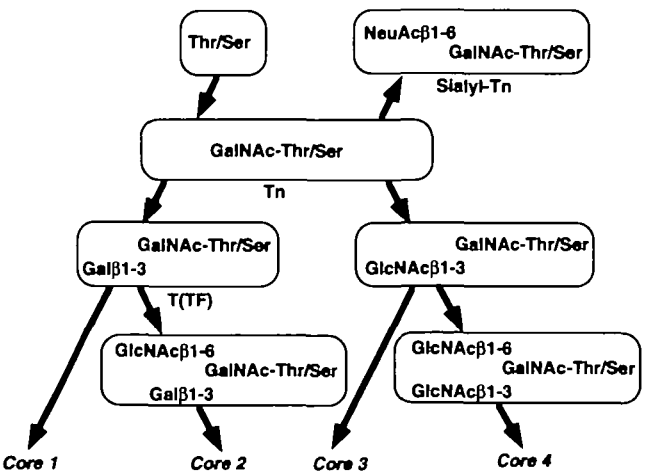

Fig. 1. **Four major types of *O*-glycan extension initiated by transfer of galactose or *N*-acetylgalactosamine.**

TABLE II. **Numbers of potential structural variations of MUC2 with 14 potential glycosylation sites within a tandem repeat.**

Within tandem repeats			Whole molecule	
Positional variations of glycosylation sites	Variations in *O*-glycan structures	Positional variations of different *O*-glycans	Genetic polymorphism	Combination of tandem repeats with different *O*-glycans
Number of variations				
Glycan or no glycan at each Thr	Core, branching, extension, and capping	Different glycan at each site	Number of tandem repeats	
2^{14}	$\sim 100^{a}$	100^{14}	51-115	Infinite

[a]A conservative estimation.

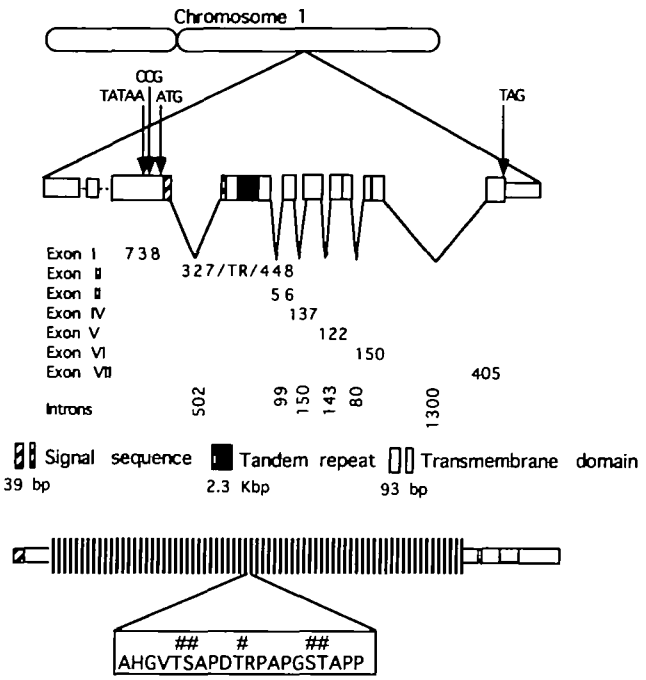

Fig. 2. **A schematic representation of MUC1 gene and mucin with an amino terminal unique domain, tandem repeat domain, transmembrane domain, and cytoplasmic domain.** MUC1 is cleaved and noncovalently reattached during processing. MUC1 is also known to be recycled and heavily sialylated after recycling. Modified from the scheme published by Gendler *et al.* (*26*).

2. Mysteries in carcinoma-associated epitopes

2.1. The PDTR epitope of MUC1. MUC1 mucin is expressed on the apical surfaces of mucous epithelial cells almost throughout the body (Fig. 2) (*26*). The binding of monoclonal antibody (mAb) specific for the core polypeptide, SM-3, and several other antibodies was apparently carcinoma-specific due to the exposure of a portion of MUC1 core peptide, the PDTR sequence (*18, 26-28*). In 1989, Finn and co-workers reported that MHC-unrestricted MUC1-specific cytotoxic lymphocytes (CTLs) can be obtained from regional lymph nodes of pancreatic carcinoma patients (*20*). CTLs with similar specificity were obtained from breast carcinoma, ovarian carcinoma, and myeloma patients (*29-32*). The CTL function was blocked by PDTR-specific monoclonal antibodies. Direct recognition of the MUC1 peptide epitope by T cell receptors in the absence of presentation by the MHC induced the same activation events that follow conventional MHC-restricted recognition (*33*). It is proposed that the highly multivalent epitopes of tandem repeats on a single MUC1 mucin molecule crosslinked the T cell receptors of MUC1-specific CTLs. The PDTR sequence apparently forms a polyproline β-turn helix type of secondary structure (*34, 35*), and the amino acid side chains radiate outward to allow their strong interactions with T cells and antibodies. Clinical trials of MUC1 core peptides in the form of synthetic peptides, vaccinia constructs, or peptides conjugated with acetylated mannan as vaccines to treat breast, pancreatic, and other carcinoma patients gave promising preliminary results. MHC class I-restricted cellular immune response to MUC1 was also observed in humans and in murine models (*36, 37*). Why humans do not develop tolerance to the PDTR epitope, which is a self-peptide and underglycosylated when the nascent peptide is synthesized in the cells, is an immunological riddle.

2.2. Truncated *O*-glycans. Clinical observations on the expression of truncated *O*-glycans and their sialylated derivatives, Tn, T, and sialyl-Tn antigens, have been reported in carcinomas from a variety of organs. These epitopes on carcinoma cells are believed to be generated by a combination of glycosyltransferases; the involvement of glycosidases has not been reported. To identify these epitopes, monoclonal antibodies that are also considered to be carcinoma markers have been used. Some monoclonal antibodies specific for T, Tn, or sialyl-Tn preferentially bind consecutive (trimeric) *O*-glycans attached to serine and threonine residues aligned on core polypeptides. Among several sialyl-Tn reactive mAbs used, B72.3 (*38*) reacted exclusively with clustered sialyl-Tn, whereas mAb B195.3R11 (*39*) reacted preferentially with unclustered sialyl-Tn. mAbs TKH2 (*40*), B239.1 (*39*), CC49 (*41*), and MLS132 (*42*) reacted more strongly with clustered sialyl-Tn than with unclustered sialyl-Tn. Studies using these antibodies suggest that sialyl-Tn displayed on carcinoma cell surfaces forms clusters along mucin core polypeptides containing consecutive serine or threonine residues (*39*). MUC2 is one of these unique core polypeptides containing consecutive and alternating threonine residues, whereas MUC1 does not contain three consecutive serine or threonine residues. For vaccine therapy, sialyl-Tn in a clustered configuration was reported to be useful (*43-45*). However, CTLs specific for sialyl-Tn have not been detected. Preliminary reports seemed to indicate that anti-Tn antibody titer showed a positive correlation with clinical outcome.

2.3. Regulation of mucin *O*-glycosylation. Many questions remain unanswered about the regulation of mucin *O*-glycosylation in carcinoma cells. Is the glycosylation change cause or result? Is the expression of a specific type of mucin core polypeptide associated with a specific type of *O*-glycan? What is the mechanism behind the poor glycosylation of MUC1 that allows the immune system to recognize the PDTR sequence? Is this mechanism related to the generation of carcinoma-associated truncated *O*-glycans such as Tn and sialyl Tn? What is the mechanism of immune response in MUC1-induced and sialyl Tn-induced host protection against carcinomas? Endeavors to answer these questions through the molecular biology of mucin core polypeptides and glycosyltransferases involved in the formation of *O*-glycans have just begun to demonstrate that diverse mucin structures are constructed under strict regulation even in carcinoma cells. Understanding the biological meaning of such a strict regulatory system that has apparently been conserved throughout the evolution of animals represents an undeveloped frontier in the field of glycobiology.

3. Diversity of MUC1 glycosylation

3.1. Structural features of *O*-glycans of MUC1. The majority of carbohydrate chains attached to MUC1 are *O*-linked through *N*-acetylgalactosamine, and their structures are often unique to a particular cell type. This range of diversity is also result of the degree of extension of the carbohydrate chains during the maturation. Many questions remain to be solved concerning the glycosylation of

MUC1. For example, it seems likely that MUC1 bears unique *O*-linked oligosaccharides that differ from those of other glycoproteins expressed on the same cells (*46*). The possible molecular functions of MUC1, discussed toward the end of this review, seem to be influenced by the pattern of glycosylation. Diversity in glycosylation of MUC1 is observed in the ratio of GalNAc-substituted threonine and serine, the patterns of glycan extension, and the patterns of peripheral epitopes (sialylation, sulfation, fucosylation *etc.*) of the glycans.

Preferential interaction of MUC1-specific mAbs and CTL clones was suggested to result from increased exposure of the peptide chain of carcinoma-associated mucins, as judged by the specificity of these antibodies, particularly mAb SM-3 (*28*). This explanation was supported by structural studies on the carbohydrate moieties of normal milk fat globular mucins and breast cancer mucins (Fig. 3) (*47-49*). Similar results were obtained by comparing

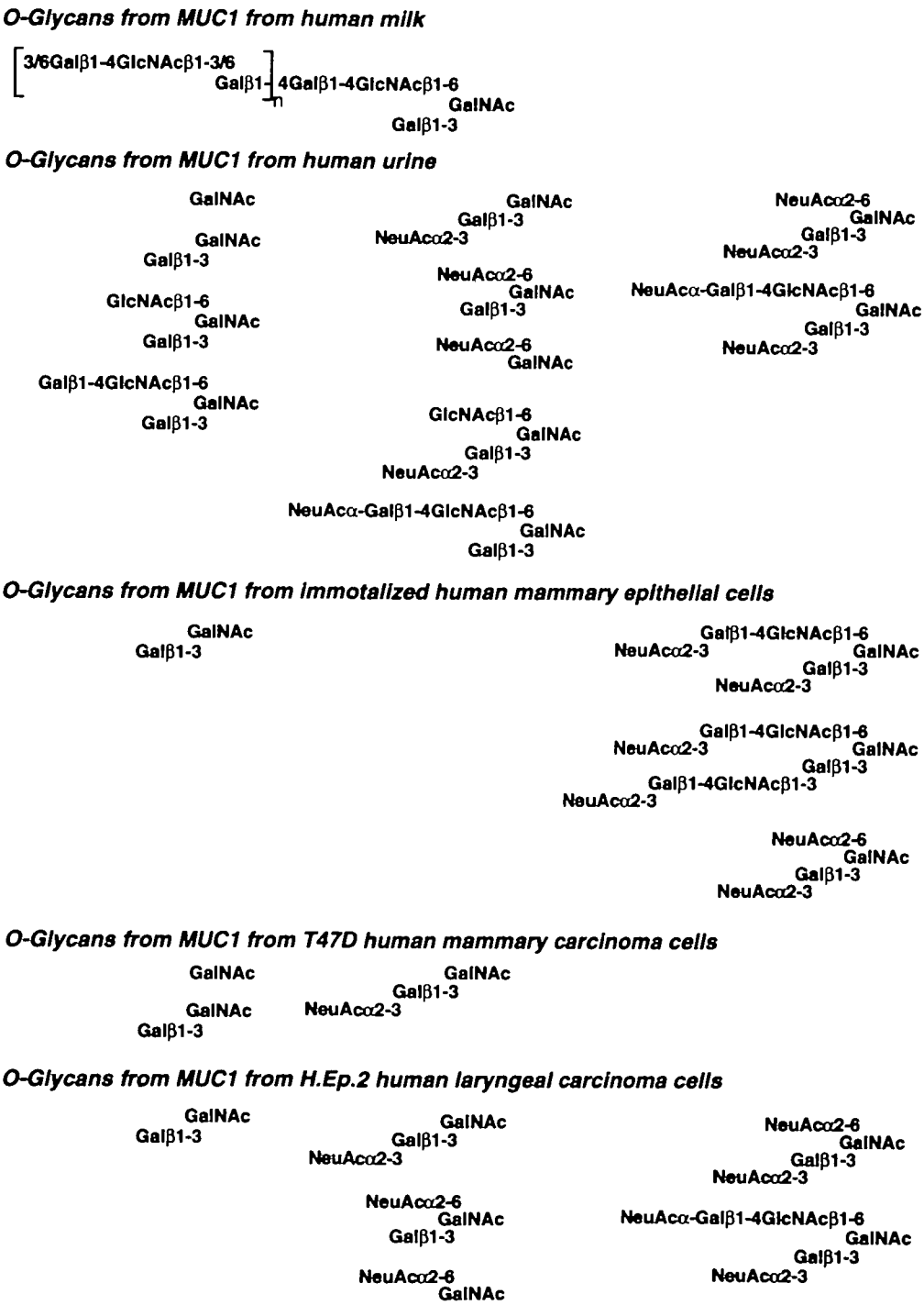

Fig. 3. Structures of *O*-glycans obtained from human milk MUC, human urine MUC1, mammary carcinoma MUC1, and laryngeal carcinoma MUC1 according to Lloyd and co-workers (*49*) and Bhavanandan and co-workers (*50*).

immortalized but non-malignant cell lines and carcinoma cell lines. Furthermore, these studies showed that normal mammary epithelial cells were able to extend *O*-glycans through the core 2 GlcNAc transferase pathway (*47-49*). This modification apparently conceals the PDTR epitope. In support of this hypothesis, cells treated with an inhibitor of *O*-glycan extensions, aryl-*N*-acetylgalactosaminide, were shown to react with some anti-MUC1 mAbs and to exhibit greater susceptibility to lysis by MUC1-specific CTLs than untreated cells (*33*). However, Bhavanandan and co-workers pointed out remarkable similarities in the total carbohydrate content, carbohydrate composition, and structures of oligosaccharides between MUC1 from urine, a non-malignant source, and human laryngeal carcinoma H.Ep.2 cells (*50*). Thus, the differential glycosylation of MUC1 between malignant and non-malignant epithelia is by no means general. Nevertheless, *O*-glycans of MUC1 are often extended through the core 2 GlcNAc transferase pathway to express peripheral epitopes such as ABO blood group antigens, Le^a and Le^x, and their sialylated and sulfated derivatives (*51*). Isolated MUC1 has been found to contain variations of extended *O*-glycans, such as sialyl Le^a, through biochemical analyses (*52*). As seen in keratan sulfate chains on MUC1 from endometrial epithelia, extended *O*-glycans on MUC1 may represent physiological regulation (*53*).

3.2. Site of *O*-glycosylation on MUC1. As stated above, it has been assumed that the peptide chain in cancer mucins has increased accessibility due to decreased glycosylation. This decrease may also be caused by other mechanisms than the extension of core 2 branching.

Müller and co-workers isolated MUC1 from human milk, partially deglycosylated by trifluoromethanesulfonic acid,

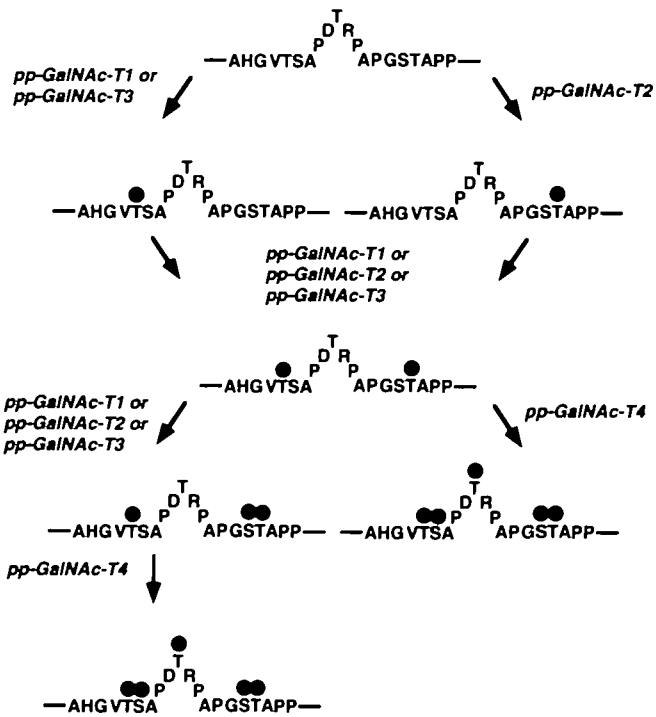

Fig. 4. **Incorporation of GalNAc into Ser and Thr within the tandem repeat of MUC1 mediated by recombinant pp-GalNAc-T1, T2, T3, and T4, according to Bennett and co-workers (*58*).**

and fragmented by endopeptidase to the size of a single tandem repeat (*48*). The glycopeptides (PAPGSTAPPAH-GVTSAPDTR with attached GalNAc residues) were then sequenced. The masses of C- or N-terminal fragments registered for the mono- to pentasubstituted glycopeptides indicated that GalNAc was linked to the peptide at Ser-5, Thr-6 (GSTA), Thr-14, Ser-15 (VTSA), and Thr-19 within the PDTR motif. The *O*-glycosylation of MUC1 tandem repeat peptides from secretory mucin of T47D breast cancer cells was also analyzed in a similar fashion. All five positions of the repeat peptides were found to be *O*-glycosylated sites, including the Thr within the PDTR motif, in this tumor cell line. Furthermore, the degree of substitution was estimated to average 4.8 glycans per repeat, higher than the 2.6 glycosylated sites per repeat for the mucin from milk (*48, 54*). This refutes the assumption of low degree of glycosylation on tumor cells based on antibody reactivity.

However, *in vitro* glycosylation of the tandem repeat portion of MUC1 gave a somewhat different view. At least eight UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (pp-GalNAc-T) genes and their protein products, which catalyze *O*-glycosylation and potentially display a distinct specificity toward peptide motifs, have been discovered. These enzymes should determine the degree and the pattern of *O*-glycosylation, which have a great impact on the accessibility of the peptide. The specificity of pp-GalNAc-Ts has been investigated in cell-free systems by the use of synthetic oligopeptides representing portions of MUC1 tandem repeats. This was done using purified enzymes in the days before cDNA cloning of these enzymes was completed. Interestingly, only Thr within the VTSA motif and/or Thr and Ser within the GSTA motif was glycosylated in cell-free systems by the enzymes from carcinoma cells (*55, 56*) or milk (*55*), despite the fact that all five putative sites including Thr in PDTR were found to be glycosylated in natural MUC1 (*48, 54*).

It is obvious that these differences could be explained by substrate specificity of the enzymes involved. Detailed studies have recently become possible through cDNA cloning and expression of pp-GalNAc-Ts. In fact, at least eight pp-GalNAc-Ts have been identified, each with a unique acceptor specificity. With GVTSAPDTRPAPGST-APPAH, a synthetic peptide similar to the one isolated by Müller and co-workers (*54*), pp-GalNAc-T1 acted preferentially at Thr-3 and Thr-15 (*56*). The initial sites preferred by pp-GalNAc-T1, T2, and T3 were Thr-3, Thr-15, and Thr-3, respectively, and neither enzyme introduced more than three GalNAc residues, at Thr-3, Ser-14, and Thr-15 (*57*).

pp-GalNAc-T4, recently cloned by Bennett and co-workers, is specific for glycopeptides into which GalNAc has been incorporated by the action of pp-GalNAc-T1-T3 (*58*). This explains how Thr and Ser residues in MUC1 are fully glycosylated with GalNAc as shown in Fig. 4. Hanisch and co-workers further proposed that the initial glycosylation of a peptide substrate influences its subsequent glycosylation (*59*). They used synthetic glycopeptides mimicking biosynthetic intermediates of MUC1 and showed that GalNAc and Galα1-3GalNAc attaching to an oligopeptide representing the MUC1 tandem repeat greatly influence the acceptor specificity of vicinal glycosylation sites. Evidence for negative effects on vicinal sites induced by

mono- or disaccharide substituents has previously been reported for a series of glycopeptide substrates based on the MUC2 tandem repeat peptide (*60*). The maximum number of incorporable GalNAc residues and the sites of GalNAc incorporation into MUC1 seem to be determined by the combination of the type of pp-GalNAc-T and the site of attachment of the initial GalNAc (Fig. 5). The biological significance of such regulation is currently unknown. It is interesting to note that the GalNAc residue attached to Thr within the PDTR motif was proposed to enhance accessibility of the core polypeptide epitope to a PDTR-specific mAb SM-3 (*61*).

In addition to modification by glycosylation, the immunodominant PDTR motif on MUC1 of T47D human mammary carcinoma cells is altered by amino acid replacements (PAPGSTAPAAHGVTSAPESR). The high incidence of these replacements and their detection in other cancer cell lines imply that the conserved tandem repeat domain of MUC1 may be polymorphic with respect to the peptide sequence (*54*).

4. Glycosylation of MUC2

4.1. Consecutive and alternating threonine residues. MUC2 is a major insoluble secreted mucin of intestinal epithelia (*5*). Its typical tandem repeat has the amino acid sequence PTTTPITTTTTVTPTPTPTGTQT. The number of tandem repeats does not seem to be highly polymorphic, whereas some replacements of amino acids are reported from one repeat to another (*62*). MUC2 is known to form insoluble oligomers through vWF-like cysteine-rich domains during biosynthesis and is also present as a cell-surface molecule of some colon carcinoma cell lines through unknown mechanisms.

As stated above, carcinoma-associated MUC2 potentially serves as a scaffold presenting a variety of carbohydrate epitopes on its abundant Thr residues. However, it is unknown how a specific arrangement of glycosylation sites is synthesized. These Thr residues are reported to be glycosylated up to 78% in the LS174T colon carcinoma cell line (*63*). *O*-Glycosylation is also initiated by a transfer of GalNAc to Thr residues by pp-GalNAc-Ts. However, variations in the arrangement of *O*-glycosylation of intestinal mucins are likely to have biological significance, particularly because MUC2 has consecutive and alternating Thr residues in the tandem repeats. To explore further the biological meaning of the consecutive and alternating Thr residues and their glycosylation, we prepared five glycopeptides with various numbers of attached GalNAc residues, corresponding to the sequences of different portions within the MUC2 tandem repeat domain. These peptides were incubated with detergent-soluble microsome fractions from human colon carcinoma cell line LS174T and UDP-GalNAc, fractionated, and characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)/peptide sequencing. An oligopeptide containing consecutive Thr residues (PTTTPITTT-T[K]) was glycosylated with 1 to 7 GalNAc residues per peptide. Sequence analysis indicated that the predominant sites incorporation of the first through sixth GalNAc residues were Thr-4, Thr-9, Thr-8, Thr-3, Thr-7, and Thr-2, respectively. An exception was a glycopeptide with three GalNAc residues at Thr-1, 4, and 5. Oligopeptides containing alternating Thr residues (TVTPTPTPTG[K] and PTPTGTQTPT[K]) were not fully glycosylated even after prolonged incubation. Thus, there was a preferential order of incorporation and a maximum number of incorporable GalNAc residues into Thr residues of MUC2 core peptides when a microsome fraction of LS174T cells was used as a source of pp-GalNAc-Ts (Iida *et al., Biochem. J.*, accepted). We subsequently found that this cell line expresses at least five pp-GalNAc-Ts. Therefore, these enzymes work in concert rather than competitively, though the mechanism remains unknown.

4.2. Regulation of GalNAc incorporation into consecutive threonines. To further clarify the biosynthetic regulation of GalNAc incorporation into consecutive Thr residues, an oligopeptide containing three consecutive Thr residues mimicking the tandem repeat portion of MUC2 (PTTTPLK) was investigated for its acceptor specificity to pp-GalNAc-T isozymes, pp-GalNAc-T1, T2, and T3. Again, the enzymatic reaction products were fractionated

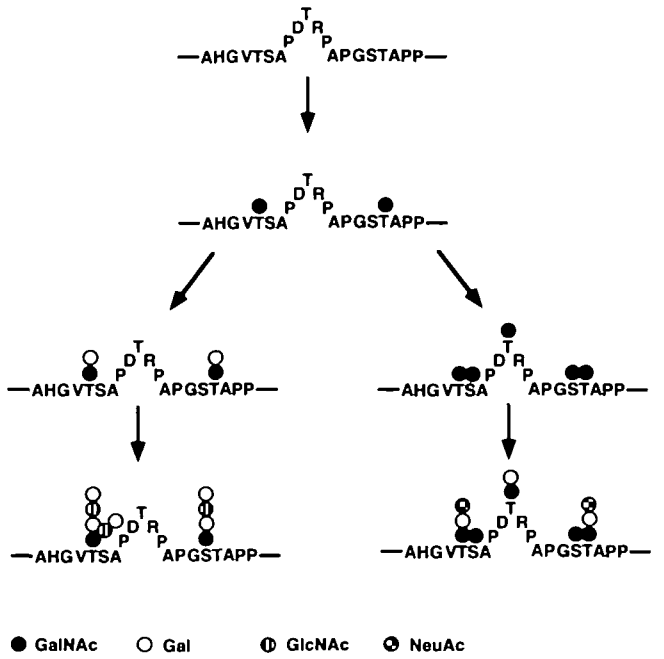

Fig. 5. **Putative regulatory mechanism of attachment of GalNAc residues and *O*-glycan extension, according to Hanisch and co-workers (*59*).**

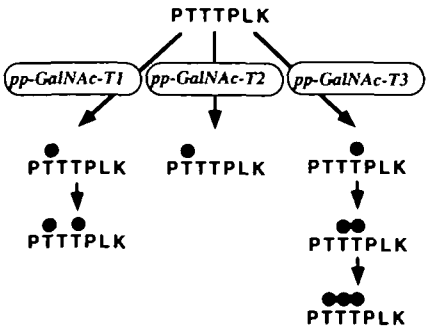

Fig. 6. **Schematic representation of the putative pathways of GalNAc incorporation into a peptide mimicking MUC2, PTTTPLK, incubated with recombinant pp-GalNAc-T1, T2, or T3.**

by reversed phase HPLC, then characterized by MALDI-TOF MS and peptide sequencing analysis. A maximum of two, one, or three GalNAc residues was transferred by pp-GalNAc-T1, T2, or T3, respectively. The preferential orders of GalNAc incorporation were Thr-2 then Thr-4 for pp-GalNAc-T1, Thr-2 for pp-GalNAc-T2, and Thr-4, Thr-3, then Thr-2 for pp-GalNAc-T3 (Fig. 6). These results indicated that the GalNAc incorporation into consecutive Thr residues in mucins was strictly regulated. It would be of interest to know whether different enzymes share common intermediates. It became obvious that there are specific mechanisms to generate consecutive *O*-glycans by a combinations of mucin core polypeptide motifs and pp-GalNAc-Ts.

5. Modulation of mucin function by altered glycosylation

5.1. MUC1 in tumor progression. It has been suggested that MUC1 has a role in tumor progression and metastasis. In colon carcinoma, primary tumors at an advanced stage and metastatic foci express increased levels of mature MUC1 (*64*). In renal cell carcinoma, sialylated MUC1 mucins are also expressed in tumors of histologically high grades or at an advanced stage, and in metastatic sites (*65*). Furthermore, MUC1 expression inversely correlated to postsurgical survival of renal cell carcinoma patients. MUC1 may play an important role in tumor growth. The growth rate of breast tumors induced by polyoma middle T antigen was found to be significantly slower in mouse homologue Muc1-deficient mice as compared with their wild-type counterparts (*66*). The tumor growth of MUC1 cDNA transfected gastric cancer cells in nude mice was increased compared to that of mock transfectant cells (*67*). However, there is a report that MUC1 cDNA-transfected colon carcinoma cells grew slower than mock transfected cells because of their anti-adhesive property (*68*). Systematic studies have not been performed to clarify the significance of glycosylation status.

5.2. Anti-adhesive and immune suppressive function of MUC1. MUC1 is a long and rigid glycoprotein that protrudes from the cell membrane. In normal glandular cells, MUC1 is expressed only on the apical (luminal) side of the cells. In most cancer cells, however, this polarization is lost, and overexpression of MUC1 on the whole cell membrane is believed to destabilize the cell-cell adhesion and permits cancer cells to migrate and metastasize (*23*). This tendency to lose homotypic cell adhesion was experimentally observed under the influence of a potent adhesion molecule, E-cadherin, as reported by Wessling and coworkers (*69*). They used double transfectant cells that expressed both MUC1 and E-cadherin to show that MUC1 could prevent intercellular adhesion mediated by E-cadherin (*69*). The length of tandem repeats is the dominant factor that determines the inhibition of E-cadherin-mediated cell-cell interactions. Furthermore, MUC1 overexpression inhibits integrin-mediated cell adhesion to extracellular matrix (*70*). This effect is reportedly observed regardless of the sialylation status of the glycans. It is also reported that MUC1 is related to the increased invasive ability of MUC1 cDNA transfected human gastric cancer cells (*67*). This effect was abolished by treatment with an inhibitor of *O*-glycan extension, benzyl-α-GalNAc. This suggests that the extracellular domain is involved in the enhancing effect of MUC1 on the motility.

MUC1 with a particular glycosylation should also function as an adhesion molecule. Sialyl Lewis X and sialyl Lewis a epitopes are found in MUC1. MUC1 with these epitopes are likely to function as ligands of carbohydrate-binding adhesion molecules, such as selectins (*52, 71-75*). Furthermore, underglycosylated MUC1 is known to be a ligand for intracellular adhesion molecule 1 (ICAM-1) (*76*). Thus, MUC1 involvement in tumor cell trafficking and interaction with host cells depends at least in part on the glycosylation.

MUC1 is known to suppress immune functions. Cells coated with MUC1 adhere less efficiently to natural killer cells, lymphokine activated killer cells, and CTLs and are resistant to their killing (*77-79*). MUC1 secreted from a colon carcinoma cell line inhibit target cell lysis by NK cells (*78*). Secreted MUC1 carrying sialyl Lewis X and sialyl Lewis a epitopes inhibit leukocyte cell adhesion to E-selectin-expressing cells (*80*). Soluble MUC1 from ascitic fluid of human breast cancer patients can inhibit the interaction through ICAM-1 (*76*). These glycosylation dependent mechanisms provide a basis for a potential link between MUC1 expression and poor prognosis.

5.3. Recognition of GalNAc clusters by lectins and antibodies. As clearly revealed in the case of sialyl-Tn epitopes, truncated *O*-glycans on carcinoma cells are detected as clusters aligned on mucin core peptides. It is widely accepted that multivalent display of carbohydrate ligands is favored by the recognition molecules in a variety of cell-to-cell recognition systems through carbohydrate-protein interactions. These include C-type lectins (*81*), galectins (*82*), and lectins expressed on the surface of microorganisms (*83, 84*). Our preliminary studies by the use of human erythrocyte glycophorin A fragments indicated that the multimeric arrangement of GalNAc residues on mucin-like molecules served as a preferential ligand for a human macrophage calcium-type lectin (hMGL) that recognizes Tn antigen (*85*).

We extended this concept to hypothesize that a specific arrangement of *O*-glycans on mucins represents a structural language like a bar code. A fluorescein-labeled synthetic peptide, PTTTPITTTTK, was converted into *O*-glycosylated glycopeptides with various numbers of attached GalNAc by *in vitro* glycosylation with UDP-GalNAc and a microsomal fraction of LS174T human colon carcinoma cells as described in Section 4.1. Glycopeptides with 1, 3, 5, and 6 GalNAc residues (G1, G3′, G5, and G6) were obtained, and their sizes were confirmed by MALDI-TOF MS. Their sequences determined by a peptide sequencer were PTTT$_{GalNAc}$PITTTTK for G1, PT$_{GalNAc}$TTPIT$_{GalNAc}$T$_{GalNAc}$TTK for G3′, PTT$_{GalNAc}$T$_{GalNAc}$PIT$_{GalNAc}$T$_{GalNAc}$T$_{GalNAc}$TK for G5, and PT$_{GalNAc}$T$_{GalNAc}$T$_{GalNAc}$PIT$_{GalNAc}$T$_{GalNAc}$T$_{GalNAc}$TK for G6. Although G3′ was previously termed as G3 (*81*), it has been re-designated as G3′ because of its glycosylation pathway. hMGL was prepared in a recombinant form, and its interaction with these glycopeptides was investigated by surface plasmon resonance spectroscopy and fluorescence polarization. The affinity of recombinant hMGL for immobilized glycopeptides was found to increase in parallel with the number of GalNAc. The highest affinity was obtained when the G6- peptide was immobilized at high density. Fluorescence polarization equilibrium-binding assays also revealed that the affinity of

hMGL for soluble glycopeptides increased depending on the number of attached GalNAcs. Carbohydrate recognition domain (CRD) fragments of hMGL were prepared, and their affinity for these four glycopeptides was also determined: this affinity was apparently lower than that of hMGL. Affinity constants of hMGL for the G3′- and G5-peptides were 11-fold and 38-fold higher, respectively, than for the G1-peptide, whereas those of CRD fragments were only 2-fold and 6-fold higher, respectively. A chemical cross-linking study revealed that hMGL, but not recombinant CRD, forms trimers in an aqueous solution. Thus, preferential binding of densely glycosylated *O*-linked glycopeptides was thought to be due to the trimer formation of hMGL. Other members of the C-type lectin family, including CD23, CD72, and asialoglycoprotein receptor are also known to form oligomers (*86, 87*). They might also show preferential binding to properly arranged *O*-glycans on mucin peptides. Immunological consequence of the effective recognition of truncated *O*-glycans by macrophages remains to be elucidated.

It is known that multimeric glycans provide a foundation for designing effective carbohydrate-based cancer vaccines (*45*) and agents interfering with microbial infection.

6. Future directions

Molecular studies on carcinoma-associated mucins have progressed a long way. Now there is no doubt that these molecules strongly influence carcinoma-host interactions in various ways. More tools, particularly cDNA, gene products, specific monoclonal antibodies to mucins, glycosyltransferases responsible for glycosylating mucins, and carbohydrate-recognition molecules interacting with mucins, are available than ever before to explore the biological meaning of diverse *O*-glycans. While tumor immunology of specific and innate immune systems is rapidly expanding, attention has also turned to mucins as regulators of pathogen interactions with epithelia.

From the viewpoint of basic biochemistry, it is exciting to realize that all Thr and Ser residues of proteins are under strict regulation with regard to the attachment of *O*-glycans. Such regulation is thought to be achieved by a combination of peptide motifs and specificity of pp-GalNAc-Ts in the case of secreted and membrane-associated proteins. Alternatively, though it is unlikely, there might be a separate compartment in the Golgi apparatus that is specialized to perform *O*-glycosylation. A new dimension to the biology of mucins has been opened with the study on MUC1 and MUC2, and its horizon is rapidly expanding to other epithelial and non-epithelial mucins.

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