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Identification of a novel cancer-specific immunodominant glycopeptide epitope in the MUC1 tandem repeat

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The cell membrane mucin MUC1 is over-expressed and aberrantly glycosylated in many cancers, and cancer-associated MUC1 glycoforms represent potential targets for immunodiagnostic and therapeutic measures. We have recently shown that MUC1 with GalNAcα1-O-Ser/Thr (Tn) and NeuAcα2-6GalNAcα1-O-Ser/Thr (STn) *O*-glycosylation is a cancer-specific glycoform, and that Tn/STn-MUC1 glycopeptide-based vaccines can override tolerance in human MUC1 transgenic mice and induce humoral immunity with high specificity for MUC1 cancer-specific glycoforms (Sorensen AL, Reis CA, Tarp MA, Mandel U, Ramachandran K, Sankaranarayanan V, Schwientek T, Graham R, Taylor-Papadimitriou J, Hollingsworth MA, et al. 2006. Chemoenzymatically synthesized multimeric Tn/STn MUC1 glycopeptides elicit cancer-specific anti-MUC1 antibody responses and override tolerance. Glycobiology. 16:96–107). In order to further characterize the immune response to Tn/STn-MUC1 glycoforms, we generated monoclonal antibodies with specificity similar to the polyclonal antibody response found in transgenic mice. In the present study, we define the immunodominant epitope on Tn/STn-MUC1 glycopeptides to the region including the amino acids GSTA of the MUC1 20-amino acid tandem repeat (HGVTSAPDTRPAPGSTAPPA). Most other MUC1 antibodies are directed to the PDTR region, although patients with antibodies to the GSTA region have been identified. A panel of other MUC1 glycoform-specific monoclonal antibodies was included for comparison. The study demonstrates that the GSTA region of the MUC1 tandem repeat contains a highly immunodominant epitope when presented with immature short *O*-glycans. The cancer-specific expression of this glycopeptide epitope makes it a prime candidate for immunodiagnostic and therapeutic measures.

Keywords: MUC1/monoclonal antibody/epitope mapping/*O*-glycosylation/cancer-specific

Introduction

Human mucin-1 (MUC1) is a large, heavily glycosylated transmembrane protein, with an extracellular region formed mainly of variable number of tandem repeats (25–125) of 20 highly conserved amino acid residues (HGVTSAPDTR-PAPGSTAPPA), each with five potential sites for *O*-glycosylation (underlined). In normal cells, MUC1 is expressed on the apical surfaces of simple and glandular epithelia and in normal breast epithelia covered with branched Galβ1-3[GlcNAcβ1-6]GalNAcα1-*O*-Ser/Thr (core 2) *O*-glycans with lactosamine extensions (Lloyd et al. 1996; Dalziel et al. 2001). In many adenocarcinomas, MUC1 is highly over-expressed and aberrantly *O*-glycosylated, leading to the exposure of immunogenic truncated glycans including the pancarcinoma carbohydrate antigens GalNAcα1-*O*-Ser/Thr (Tn), NeuAcα2-6GalNAcα1-*O*-Ser/Thr (STn), and Galβ1-3GalNAcα1-*O*-Ser/Thr (T) (Springer 1984; Taylor-Papadimitriou and Epenetos 1994). In breast cancer patients, the presence of circulating antibodies directed to the under-glycosylated MUC1 tandem repeat peptide sequence is correlated with a better prognosis (Rughetti et al. 1993; von Mensdorff-Pouilly et al. 2000a, 2000b), and CD8+ T cells specific for MUC1 peptides have been identified in the peripheral blood of breast cancer patients (Feuerer et al. 2001; Correa et al. 2005). Hence, MUC1 has long been considered a target for immunodiagnostic and therapeutic measures in adenocarcinoma patients (Taylor-Papadimitriou and Epenetos 1994; Samuel and Longenecker 1995).

In the past, a large number of monoclonal antibodies (MAbs) have been produced to purify MUC1 and synthetic peptides and glycopeptides derived from MUC1. The epitopes of these MAbs have traditionally been defined by scanning overlapping short peptides, and most of the MAbs define epitopes in the heavily *O*-glycosylated mucin tandem repeat domain. One large group of MAbs have been raised against human milk fat globule (HMFG) including HMFG1 (Taylor-Papadimitriou et al. 1981), 115D8 (Hilkens et al. 1984), and SM3 (Burchell et al. 1987), most of which react with an epitope in the PDTR region of the MUC1 tandem repeat considered to be the immunodominant peptide epitope in wildtype mice. Only a few MAbs defining tandem repeat epitopes outside the PDTR region have been reported. One generated against breast cancer tissue extract, DF3, is used in the CA 15-3 screening assay in combination with 115D8 and defines the peptide epitope TRPAPGS (Kufe et al. 1984). Immunization with unglycosylated MUC1 peptide has given

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rise to a low-affinity MAb (BCP9) reactive with the GSTAP peptide (Xing et al. 1992).

Most MUC1 antibodies react with the peptide backbone, but often the binding is modulated by the presence of glycans. In some cases, the presence of a particular glycan can enhance binding as seen with B27.29 (Grinstead et al. 2002; Grinstead et al. 2003), 115D8 (Dai et al. 1998), and VU-2-G7 (Ryuko et al. 2000). In other examples, glycans can inhibit binding, as seen with SM3 and HMFG1. SM3 was raised against chemically deglycosylated HMFG and exhibits high preference for cancer-associated MUC1—opposed to other MAbs raised against HMFG—because the antibody binding to the PDTR region is selectively blocked by large-branched *O*-glycans as found in normal breast epithelium (Burchell et al. 2001).

A few antibodies reacting specifically with MUC1 glycoforms have been reported. One MAb, BW835, was generated by alternating injections of cancer cell lines MCF-7 and SW-613, and the specificity is reported to be restricted to the glycopeptide epitope VTSA, where Thr is substituted with the T antigen (Hanisch et al. 1995). The MAb MY.1E12 (Yamamoto et al. 1996) was raised against HMFG and the epitope maps to the same peptide sequence, but here sialylation of the T structure (rendering the ST structure) enhances reactivity (Takeuchi et al. 2002). Recently, we found that immunization with long Tn- or STn-MUC1 tandem repeat glycopeptides can override tolerance in humanized MUC1 transgenic Balb/c mice (Sorensen et al. 2006). The humoral immune response induced with the glycopeptide vaccines was highly specific for the Tn/STn-MUC1 glycoforms and MUC1 expressed by human cancer cells. In order to further characterize immunity to these glycopeptides, we generated MAbs that mimic the polyclonal response elicited in MUC1 transgenic mice.

In the present study, we report on the fine specificity of anti-MUC1 antisera and MAbs (5E5 and 2D9) raised against MUC1 Tn/STn glycopeptides. By using a panel of different well-defined MUC1 tandem repeat glycopeptides, we show in direct binding and streptavidin–biotin capture enzyme-linked immunosorbent assay (ELISA) that the reactivity of 5E5 and 2D9 is restricted to MUC1 glycopeptides, with Tn- or STn-glycosylation in the GSTA region. The same specificity was found with total serum antibodies from human MUC1 transgenic mice vaccinated with Tn-MUC1 glycopeptide. The study defines a novel immunodominant glycopeptide epitope in the MUC1 tandem repeat, which is specifically expressed in cancer.

Results

Generation of MUC1 MAbs

The MAb 5E5 (IgG_1) was raised against 60-mer MUC1 tandem repeat peptide carrying 15 *N*-acetylgalactosamine (GalNAc) residues conjugated to KLH as described previously (Sorensen et al. 2006). This antibody was shown to specifically react with MUC1 carrying Tn or STn in the tandem repeat domain and reacts with the vast majority of breast carcinomas, although showing no reactivity with normal breast epithelia (Sorensen et al. 2006). 5E5 was originally selected because its reactivity pattern essentially mirrored that of total sera from MUC1 transgenic mice immunized with MUC1 tandem repeat glycopeptides with complete Tn- or STn-glycosylation (Sorensen et al. 2006). In the present study, we have reproduced the immunization and screening protocol and isolated another MAb, 2D9 (IgG_1), which exhibits essentially the same specificity, demonstrating that such antibodies are prevalent.

Two additional MUC1 antibodies were raised against purified recombinant secreted MUC1 (rMUC1) expressed in CHO ldlD cells grown in GalNAc to produce the Tn glycoform (5E10) or grown in galactose (Gal) and GalNAc to produce the ST glycoform, which after neuraminidase treatment was reduced to the T glycoform (1B9). By immunocytochemistry, MAb 5E10 reacted with all the MUC1-expressing cell lines tested and therefore potentially could serve as a universal anti-MUC1 MAb. MAb 1B9 was selected because it showed specificity for neuraminidase-treated cells presenting the T glycoform of MUC1 (data not shown).

Epitope mapping of MAbs 5E5 and 2D9 raised against Tn-MUC1 tandem repeat glycopeptides

The specificity of the antibodies was initially determined by direct-binding ELISA using a panel of 60-mer glycopeptides produced by chemoenzymatic methods (Figure 1) (Sorensen et al. 2006). The MAbs 5E5 and 2D9 exhibited a similar reactivity pattern with high selectivity for MUC1 tandem repeat glycopeptides with Tn and STn *O*-glycans and both antibodies showed preference for Tn-MUC1 glycoforms with highest *O*-glycan occupancy; however, in direct-binding assays, 2D9 showed significantly better reactivity with Tn glycoforms compared with STn glycoforms (not shown). In order to fully assess the binding specificity of the antibodies and eliminate issues with differences in adsorption and presentation of MUC1 peptides and glycopeptides in direct-binding ELISA, a streptavidin–biotin capture ELISA was developed using a large panel of 60-mer-based MUC1-biotinylated glycopeptides (Figure 1). The results shown in Figure 2 clearly confirm that the two MAbs, 5E5 and 2D9, react with a glycopeptide epitope where the glycan can be Tn or STn, with at least two *O*-glycans and preferably three or five *O*-glycans per MUC1 repeat. This could suggest that an *O*-glycan in either the VTSA or the GSTV region of the sequence is required for the epitope (Figure 1). Recombinant expression of MUC1 in CHO ldlD cells allows presentation of different glycoforms of MUC1 (Sorensen et al. 2006). MAbs 5E5 and 2D9 reacted with Tn-MUC1 but not with T-MUC1 glycoforms, as predicted by the lack of reactivity with T and ST-MUC1 glycopeptides (Figure 2A and D). Interestingly, weak reactivity was observed with the core 3 *O*-glycosylated glycoform (GlcNAcβ1–3GalNAcα1-*O*-Ser/Thr) but only when this is presented with three and not with five *O*-glycans per tandem repeat. This may be due to a minor residual amount of partially β3GlcNAc-substituted glycopeptide, as suggested by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of this glycopeptide (data not shown).

Further glycopeptide variants are required to more precisely define the epitopes; however, present enzymatic glycosylation of 60-mer MUC1 peptides are limited by the substrate specificities of polypeptide GalNAc-transferases (Wandall et al. 1997; Bennett et al. 1998). Therefore, two

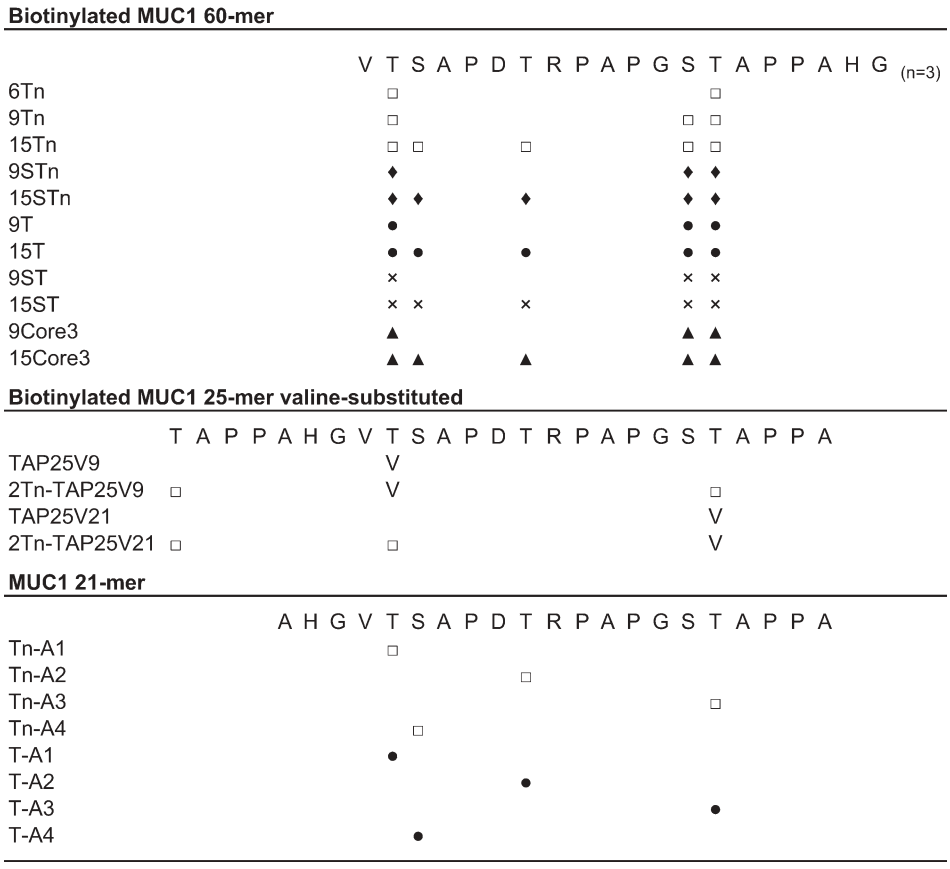

Fig. 1. Glycopeptides used for the characterization of MAb specificities. Biotinylated 60-mer glycopeptides: prefix numbers indicate number of *O*-glycans in peptides. Biotinylated 25-mer valine-substituted glycopeptides: TAP25V9, valine-substituted in position 9; TAP25V21, valine-substituted in position 21; 2Tn-TAP25V9 and 2Tn-TAP25V21 are glycosylated with Tn in the indicated positions. 21-mer: synthetic glycopeptides with a single Tn or T glycan in the indicated positions.

25-mer peptides with valine substitutions of selected threonine residues were used to chemoenzymatically produce glycoforms with Tn at individual sites utilizing GalNAc-T11 (Figure 1) (Hassan et al. 2000; Schwientek et al. 2002). Besides Tn-glycosylation of the initial Thr, the two glycopeptides were Tn-glycosylated at Thr either in the VTSA region (2Tn-TAP25V21) or in the GSTA region (2Tn-TAP25V9). Enzymatic Tn-glycosylation of both Ser and Thr in the GSTA region, which was observed to increase reactivity with the biotinylated 60-mer peptide, was not possible with the TAP25V9 peptide (Wandall et al. 1997; Hassan et al. 2000). As shown in Figure 2B and E, 5E5 and 2D9 did not react with 2Tn-TAP25V21, with Tn-glycosylation at Thr in the VTSA region, whereas strong reactivity was found with the 2Tn-TAP25V9 glycopeptide, with Tn-glycosylation at Thr in the GSTA region. This reactivity pattern was confirmed in direct-binding ELISA with a panel of synthetic MUC1 glycopeptides with one single Tn or T *O*-glycan (Karsten et al. 1998) (Figure 1). 5E5 and 2D9 showed strong reactivity towards the glycopeptide, with Tn at the Thr in the GSTA region, whereas no reactivity was seen when the T glycan was carried on this threonine or with the other Tn- or T-glycosylated glycopeptides (Figure 2C and F). In summary, 5E5 and 2D9 reacted with MUC1 glycopeptides when Thr in GSTA is Tn- or STn-glycosylated and stronger when both Ser and Thr are glycosylated.

Specificity analysis of total serum from Tn-MUC1-immunized MUC1 transgenic mice

Total serum of mice immunized with the 15Tn-MUC1 60-mer glycopeptide conjugated to KLH showed the same preference for high-density Tn- and STn-MUC1 glycopeptides (Figure 3A). More importantly, the same specificity for the Tn-glycosylated GSTA sequence was observed with the valine-substituted glycopeptides (Figure 3B). Taken together, these results clearly indicate that the GSTA region of the MUC1 tandem repeat glycosylated with Tn and/or STn represents a novel immunodominant MUC1 glycopeptide epitope.

Characterization of MAbs 1B9 and 5E10

The two MAbs raised against recombinant MUC1 glycoprotein expressed in CHO ldlD cells were analyzed with the capture ELISA, using the panel of MUC1 60-mer biotinylated glycopeptides. MAb 1B9 showed strong reactivity with the MUC1 glycopeptide with three T *O*-glycans per tandem repeat, whereas only an extremely weak reaction was observed with the glycopeptide fully substituted with five T *O*-glycans per tandem repeat (Figure 4A). Intermediate reactivity was seen with peptides substituted with core 3 and ST, but only with peptides with three glycans per tandem repeat (Figure 4A). ELISA with MUC1 glycopeptides with one

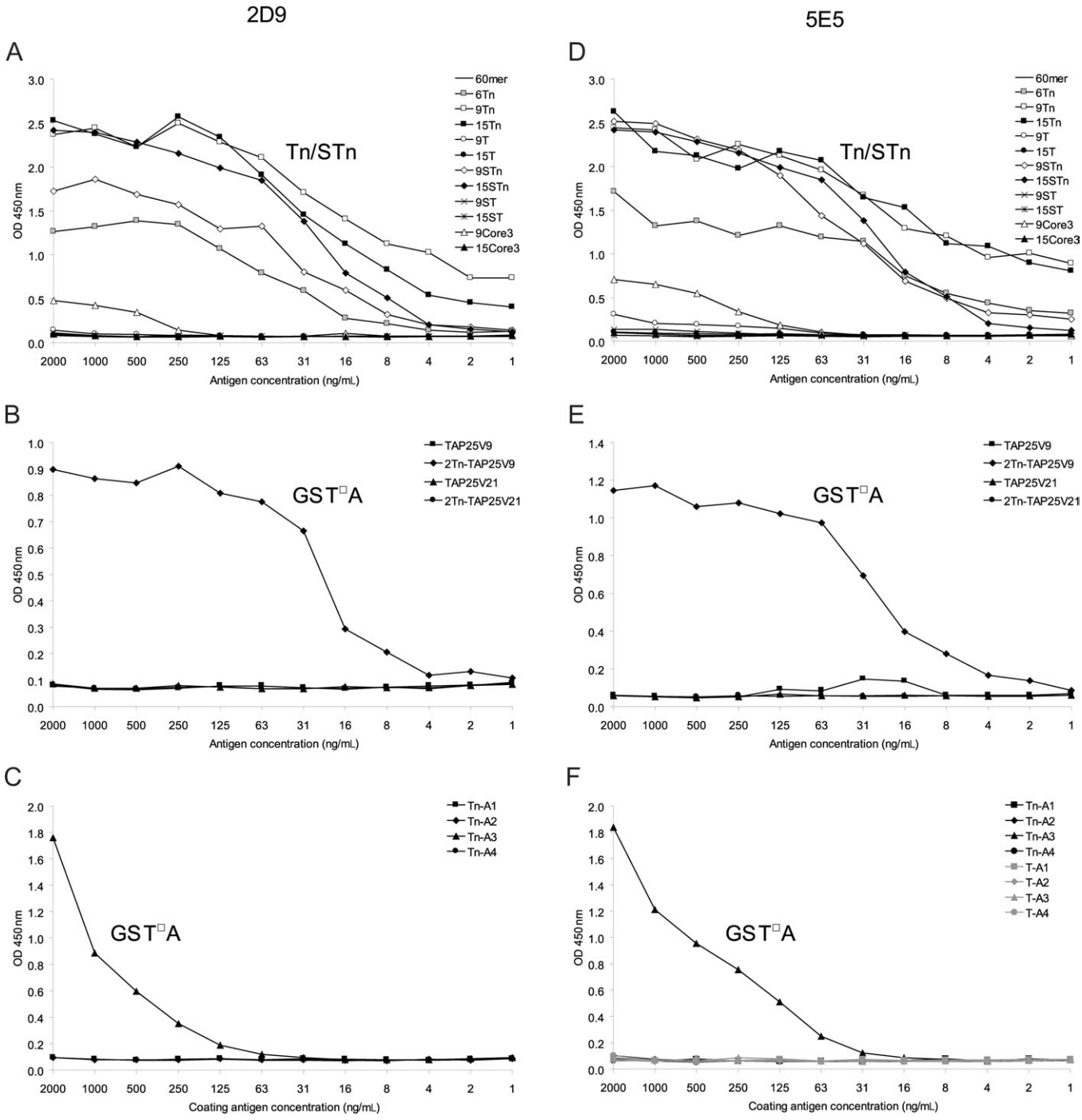

Fig. 2. Specificity analysis of MAbs 2D9 and 5E5 by ELISA. [(**A**) and (**D**)] Reactivity of MAbs 2D9 and 5E5 with biotinylated 60-mer glycopeptides by capture ELISA (Figure 1). Strong reactivity is seen for both MAbs with high-density Tn and STn glycoforms. [(**B**) and (**E**)] Reactivity of MAbs 2D9 and 5E5 with biotinylated valine-substituted 25-mer glycopeptides by capture ELISA. Strong reactivity is seen for both MAbs with the peptide Tn-glycosylated at Thr in the GSTA region. □ indicates Tn-glycosylation. [(**C**) and (**F**)] Reactivity of MAbs 2D9 and 5E5 with 21-mer glycopeptides glycosylated with a single Tn or T glycan by direct-binding ELISA. Strong reactivity is seen for both MAbs, with the peptide Tn-glycosylated at Thr in the GSTA region. □ indicates Tn-glycosylation. Controls for 25- and 21-mer peptides are shown with MAb 5E10 in Figure 5B and C.

single T *O*-glycan showed reactivity with the peptide T-glycosylated at Thr in the amino acid sequence GSTA, but also, although weaker, with the peptide T-glycosylated at Thr in the amino acid sequence VTSA (Figure 4B). No reactivity was seen with the MUC1 glycopeptides with one single Tn *O*-glycan. Furthermore, 1B9 did not react with CHO ldlD MUC1-expressing cells when grown in GalNAc alone, but only when Gal is added to the growth medium, leading to expression of ST-MUC1. Significantly enhanced reactivity was seen after neuraminidase treatment of the cells to expose T-MUC1 (Figure 4C). These data suggest that the epitope for 1B9 is not a glycopeptide epitope, but rather a conformational

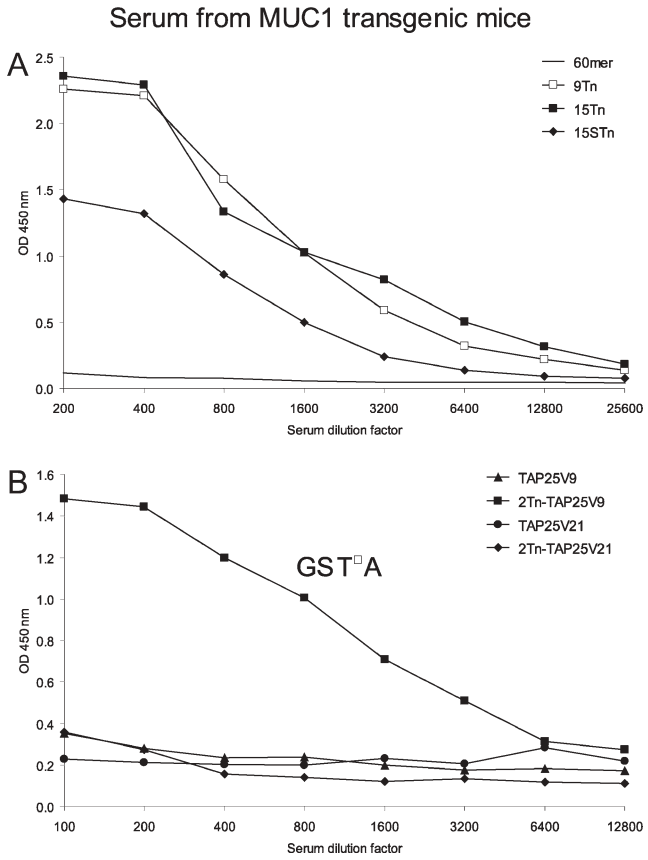

Fig. 3. Specificity analysis of serum from MUC1 transgenic mice immunized with 15Tn-MUC1 60-mer glycopeptide. (**A**) Reactivity with 60-mer glycopeptides by direct-binding ELISA. Strong reactivity is seen with glycopeptides with three or five Tn glycans per tandem repeat. Lower reactivity is seen with the fully STn-glycosylated peptide. No reactivity is seen with the unglycosylated peptide. (**B**) Reactivity with biotinylated valine-substituted 25-mer glycopeptides by capture ELISA. Strong reactivity is seen with the peptide Tn-glycosylated at Thr in the GSTA region. □ indicates Tn-glycosylation.

epitope requiring glycosylation with β1-3-linked disaccharides (T or core 3) in either the VTSA or the GSTA regions, but not the PDTR region.

The MAb 5E10 showed highest reactivity with biotinylated MUC1 60-mer peptide or when the peptide is either unglycosylated or substituted with only two Tn glycans per tandem repeat. Reactivity towards Tn-glycosylation decreased with increasing density of glycosylation. An additional decrease in reactivity was seen with the introduction of T-glycosylation, followed by a further decrease by introduction of core 3 glycosylation. Lowest reactivity was seen with increasing degrees of sialylation, especially when sialic acid (NeuAc) is α2-6-linked to GalNAc (STn). No reactivity at all was seen with the peptide fully substituted with STn (Figure 5A). In ELISA with biotinylated, valine-substituted, in vitro Tn-glycosylated MUC1 peptides, equal reactivity was seen with all four peptides regardless of glycosylation (Figure 5B). In ELISA with MUC1 glycopeptides with one single Tn or T *O*-glycan, equal reactivity was seen with all peptides except for the peptide T-glycosylated at Thr in the amino acid sequence GSTA (Figure 5C). In immunocytology, 5E10 reacted independently with CHO ldlD MUC1-expressing cells on co-culturing with GalNAc, Gal, or no sugar at all (data not shown). In summary, 5E10 reacted with all MUC1 glycoforms tested with the exception of complete STn occupancy.

Comparison with other MAbs previously reported to react with MUC1 glycoforms

The MAb SM3 binds the PDTR region of the MUC1 tandem repeat, and Tn-glycosylation of the Thr enhances its binding (Burchell et al. 1989; Dokurno et al. 1998). In agreement with previous reports, SM3 preferentially reacted with the unglycosylated peptide and the glycopeptides with complete *O*-glycan occupancy of five *O*-glycans per tandem repeat, and reactivity with glycopeptides with two and three *O*-glycans was lower (Figure 5D) (Reis et al. 1998). These results confirm and extend our previous studies to demonstrate that T, ST, and core 3 *O*-glycans react equally well. For this study, we did not have core 2 glycoforms, but studies with cell lines clearly indicate that core 2 glycosylation of MUC1 blocks the SM3 epitope (Beum et al. 1999; Dalziel et al. 2001; Mungul et al. 2004). Little reactivity was observed with the biotinylated, valine-substituted peptides, whether unglycosylated or with Tn-glycosylation of Thr in the amino acid sequences VTSA or GSTA (Figure 5E). In ELISA with MUC1 glycopeptides with one single Tn or T *O*-glycan, high reactivity was seen when Thr in the amino acid sequence PDTR is substituted with either T or Tn. Lower reactivity was seen with the remaining T-MUC1 glycopeptides, whereas hardly any reactivity was observed with the remaining Tn-MUC1 glycopeptides (Figure 5F).

MAb BW835 reacted in the capture ELISA with biotinylated MUC1 60-mer glycopeptides fully glycosylated with the disaccharides T (Figure 4C). Interestingly, BW835 reacted equally well with the core 3 *O*-glycosylated peptide, indicating that the antibody does not require the T-MUC1 glycoform per se but rather a β1-3-linked disaccharide in the VTSA region of MUC1. Lower reactivity was also found with the fully ST-glycosylated peptide. Weak reactivity was observed with the T glycopeptide with only three *O*-glycans, and similar weak reactivity was found with the fully Tn-glycosylated glycopeptide. No reactivity was found with glycopeptides carrying two or three Tn glycans per TR, STn-glycosylated, or unglycosylated peptides. These results are in agreement with and extend previous characterization of the epitope.

In accordance with earlier published data (Takeuchi et al. 2002), MY.1E12 showed strict specificity for the ST-glycoforms of MUC1, as evaluated with biotinylated 60-mer glycopeptides (Figure 4D). In contrast to BW835, MY.1E12 showed preference for the peptide with three ST *O*-glycans per tandem repeat, suggesting that the epitope is at least partially destroyed when both Thr and Ser in the VTSA region are glycosylated.

Discussion

The primary objective of this study was to define the unique glycopeptide epitope of antibodies elicited in wildtype and human MUC1 transgenic mice to large Tn- and STn-MUC1 60-mer glycopeptides. In our previous studies, we defined a MUC1 glycopeptide vaccine capable of overriding tolerance

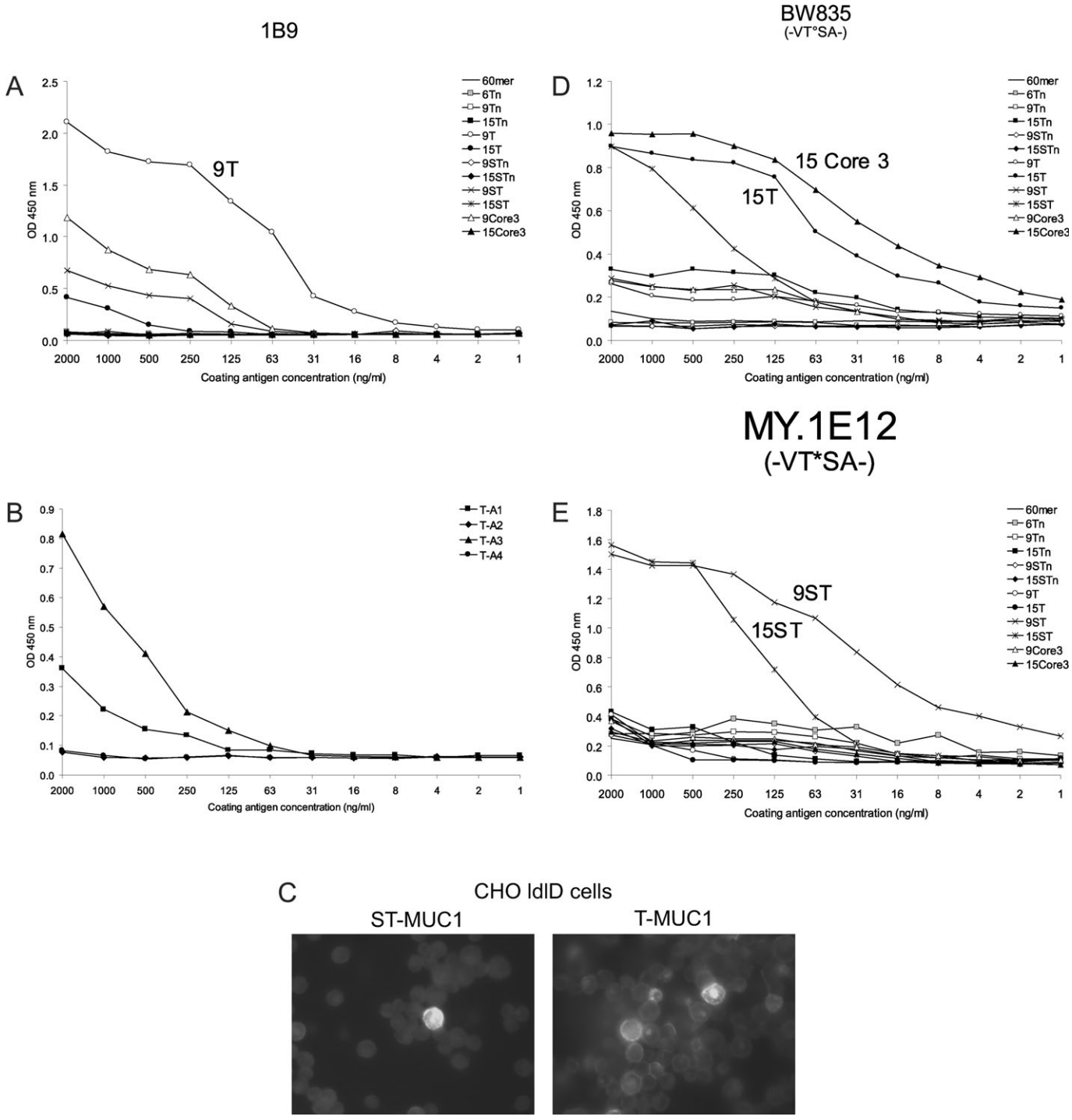

Fig. 4. Specificity analysis of MAbs 1B9, BW835, and MY.1E12 by ELISA. (**A**) Reactivity of MAb 1B9 with biotinylated 60-mer glycopeptides. Reactivity is also seen with core 3 and ST glycans (see text for details). (**B**) Reactivity of MAb 1B9 with biotinylated valine-substituted 25-mer glycopeptides. Reactivity is seen with glycopeptides, with T glycans at Thr in the GSTA region and Thr in the VTSA region. (**C**) Reactivity of MAb 1B9 with CHO ldlD cells grown in Gal/GalNAc. 1B9 reacts with approximately 2% of the cells expressing ST-MUC1 (no neuraminidase treatment), whereas it reacts with approximately 20% of cells presenting T-MUC1 (neuraminidase treated). (**D**) Reactivity of MAb BW835 with biotinylated 60-mer glycopeptides. Published epitope listed in parentheses, where ○ indicates T-glycosylation. Strong reactivity is seen with the glycopeptides with five T or core 3 glycans per tandem repeat. Lower reactivity is observed with five ST glycans per tandem repeat. (**E**) Reactivity of MAb MY.1E12 with biotinylated 60-mer glycopeptides. Published epitope listed in parentheses, where * indicates ST-glycosylation. Strong reactivity is seen with the glycopeptide with five ST glycans per tandem repeat. Lower reactivity is observed with three ST glycans per tandem repeat.

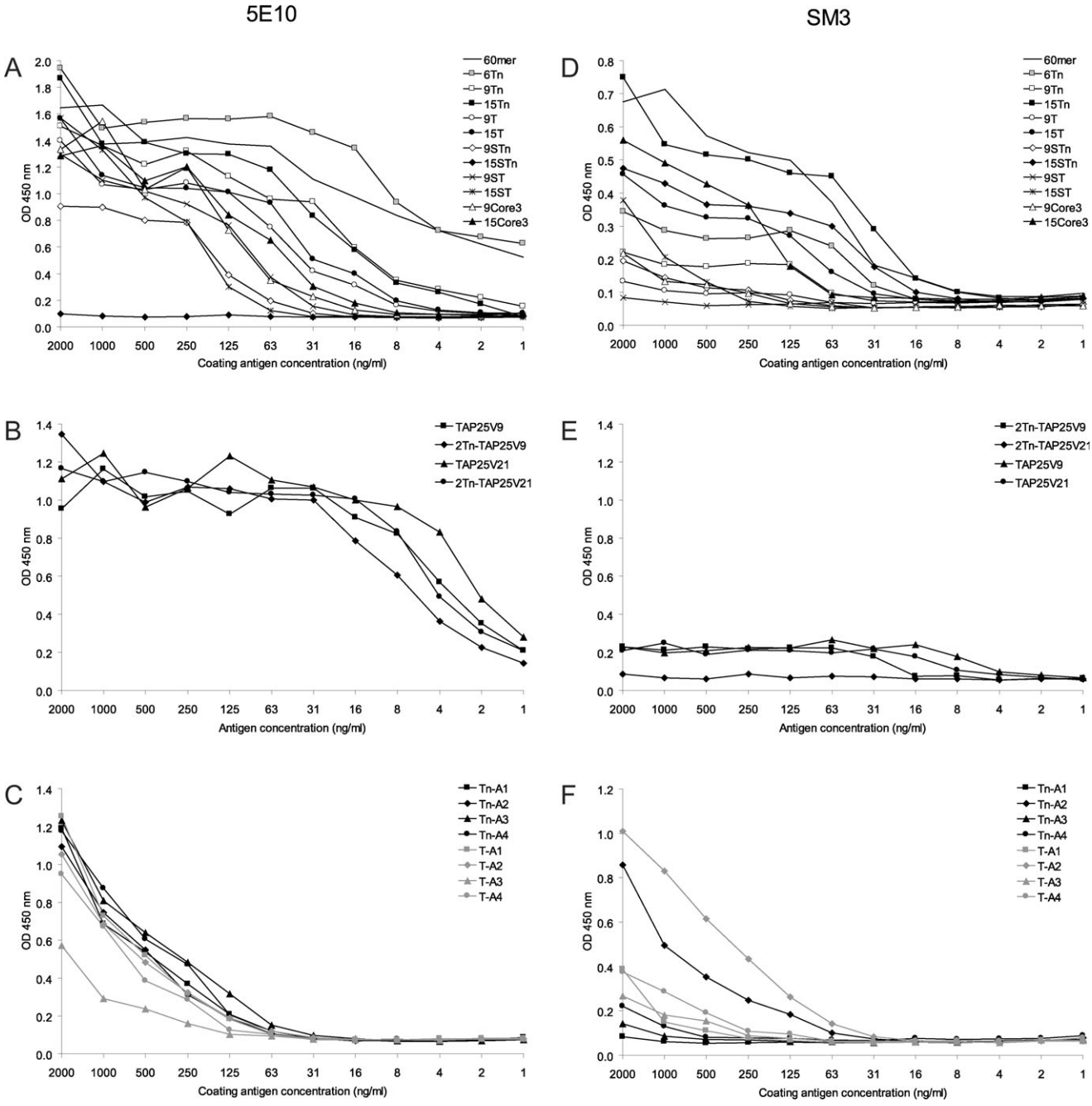

Fig. 5. Specificity analysis of MAbs 5E10 and SM3 by ELISA. (**A**) Reactivity of MAb 5E10 with biotinylated 60-mer glycopeptides. Reactivity is seen with all tested peptides except when fully glycosylated with the STn glycan. Preference is seen for unglycosylated and Tn glycoforms, followed by T and ST glycoforms. Lowest reactivity is seen with the glycopeptide with three STn glycans per tandem repeat. (**B**) Reactivity of MAb 5E10 with biotinylated valine-substituted 25-mer glycopeptides. Strong reactivity is seen with all peptides independent on Tn-glycosylation. (**C**) Reactivity of MAb 5E10 with 21-mer glycopeptides glycosylated with a single Tn or T glycan. Relatively strong reactivity is seen with all glycopeptides with exception of the peptide with a T glycan at Thr in the PDTR region. (**D**) Reactivity of MAb SM3 with biotinylated 60-mer glycopeptides. Strongest reactivity is seen with unglycosylated peptide or peptides with five *O*-glycans per tandem repeat. The glycoforms of preference are Tn, STn, core 3, T, and ST in the mentioned order. (**E**) Reactivity of MAb SM3 with biotinylated valine-substituted 25-mer glycopeptides. Weak reactivity is seen with the unglycosylated peptides and where the Thr in the GSTA region is Tn-glycosylated. No reactivity is seen with the glycopeptide glycosylated in the Thr in the VTSA region. (**F**) Reactivity of MAb SM3 with 21-mer glycopeptides glycosylated with a single Tn or T glycan. Strongest reactivity is seen with glycopeptides with T- or Tn-glycosylation at Thr in the PDTR region. Low reactivity is seen with the remaining T-glycosylated and some of the Tn-glycosylated glycopeptides.

in human MUC1 transgenic mice and eliciting highly cancer-specific MUC1 immunity (Sorensen et al. 2006). Further defining the epitope is important for understanding the underlying molecular mechanism for biosynthesis and expression of the antigen and may aid in further optimization of vaccine designs. A novel glycopeptide epitope was identified in the GSTA region of the MUC1 tandem repeat, and both MAbs and total sera from immunized MUC1 transgenic mice were

directed almost exclusively to this epitope. The epitope was extensively defined with respect to requirements for glycoform and site occupancy and limited essentially to the Tn and STn structures in the GSTA region of the tandem repeat of MUC1. Past studies have defined the PDTR region as the major immunodominant epitope of unglycosylated MUC1 tandem repeat peptides in the mouse, as the vast majority of mouse MAbs generated to MUC1 are directed to this region. All but a few exceptions, with SM3 being the most notable example, do not exhibit cancer-associated reactivity patterns. Interestingly, our studies with immunization of MUC1 glycopeptides failed to show antibodies towards the PDTR region in wildtype mice as well as in human transgenic mice. Mouse MAbs directed against the glycosylated GSTA region have not been found previously using different MUC1-based immunogens (Xing et al. 2001). Thus the results described here suggest that the Tn/STn glycopeptide epitope in the GSTA region represents a novel, valuable, immunodominant, mixed glycopeptide epitope with significant immunodiagnostic and therapeutic potential.

Many MUC1 antibodies with epitopes in the PDTR region are affected by *O*-glycosylation of Thr in the PDTR region as well as in either of the sites in the VTSA or GSTA regions. The binding of the antibody SM3 to the PDTR region of MUC1 has been studied in detail by X-ray crystallography and NMR spectroscopy (Dokurno et al. 1998; Moller et al. 2002), and GalNAc *O*-glycosylation of the Thr residue in PDTR induces conformational changes in the peptide region, enhancing antibody interaction with the peptide core, but without siginificant interaction with the GalNAc residue. Similarly, binding of the MAb C595 to the RPAP peptide epitope is enhanced by conformational changes induced by Tn-glycosylation of Thr in VTSA as well as Ser and Thr in GSTA regions (Spencer et al. 1999). In contrast, NMR studies of the binding of the antibody B27.29 suggest that the antibody epitope maps to two separate parts of the glycopeptide, with the core peptide epitope spanning the PDTRP sequence and a second carbohydrate epitope comprising Tn-glycans at Thr and Ser in the VTSA region (Grinstead et al. 2002). The antibodies 5E5 and 2D9 and sera from 15Tn-MUC1-immunized MUC1 transgenic mice do not bind unglycosylated MUC1 peptides and exhibit high selectivity for glycoform (Tn and STn) and site of glycosylation (GSTA region), indicating that the antibody-binding site involves both peptide and carbohydrate moieties. It is quite surprising that antibodies to glycoforms in the GSTA region have not been previously identified considering that Tn and STn glycoforms of MUC1 are highly overexpressed in cancer cells (Sorensen et al. 2006). Cancer patients have been found to have antibodies to Tn glycopeptides, although the fine specificity of these have not been determined (von Mensdorff-Pouilly et al. 2000a). Human antibodies to peptide epitopes in the RPAP and APPAH regions have also been mapped (Nuti et al. 1992; Petrarca et al. 1996).

In preliminary studies, we have assessed immunogenicity of rMUC1 expressed in CHO ldlD cells with Tn (Tn-rMUC1) or T (T-rMUC1) glycosylation. Interestingly, Tn-rMUC1 elicited antibody responses in wildtype mice directed to both unglycosylated and glycosylated MUC1 tandem repeat (glyco)peptides (not shown), and thus not similar to the immune response observed with Tn-MUC1 glycopeptide immunization. One MAb, 5E10, with representative specificity was selected from mice immunized with Tn-rMUC1 for further characterization, and the specificity of this antibody was found to be essentially similar to antibodies such as HMFG1 and 2 (Taylor-Papadimitriou et al. 1981). It is presently unclear why Tn-rMUC1 did not induce Tn glycopeptide-specific antibodies, but it may be related to lower *O*-glycan density of the tandem repeat region of rMUC1 expressed in CHO ldlD cells. Secreted rMUC1 expressed in CHO K1 cells was found to have 4.3 *O*-glycans per repeat (Backstrom et al. 2003), and the density of *O*-glycans in CHO ldlD is expected to be lower. Immunogenicity of T-rMUC1 was poor (not shown), but one MAb, 1B9, specifically reacting with T-MUC1 glycopeptides was selected. Epitope mapping suggested that the epitope was likely conformational, because there was no absolute requirement for T-glycosylation at a particular site, but rather T-glycosylation in either the VTSA or the GSTA regions was sufficient for binding. Further studies of vaccines based on secreted rMUC1 are clearly required, but we believe it is essential to use any of the cancer-associated glycoforms Tn, STn, and T, which makes CHO ldlD cells ideal. In this respect, we have recently shown that rMUC1 expressed in CHO K1 cells with ST-glycosylation, which is a normal glycoform found on many cells, in fact, may serve immunosuppressive functions because it impairs the differentiation and function of dendritic cells (Rughetti et al. 2005).

Human immunity to MUC1 tandem repeat-derived peptides has been explored in some detail. Karanikas et al. (1997) found that immunizing cancer patients with a mannan-MUC1 fusion protein elicited strong antibody responses in 13 of 25 patients directed against the STAPPAHG and PAPGSTAP regions of unglycosylated MUC1 tandem repeat peptides. These antibodies, however, did not react with MUC1 expressed by human cancer cells and hence are likely to be blocked by *O*-glycosylation. The same group has recently reported on a pilot phase III study with early-stage breast cancer patients receiving the mannan-MUC1 vaccine (Apostolopoulos et al. 2006). None of the vaccinated patients had recurrence, and 9 of 13 vaccinated patients developed MUC1-specific antibodies, whereas the control group receiving placebo had 27% recurrence and no immune responses to MUC1. Whether the generated MUC1 antibodies reacted with cancer-associated MUC1 is unknown. Gilewski et al. (2000) found that a KLH-conjugated 30-mer unglycosylated MUC1 tandem repeat peptide induced high IgM and IgG titers reactive with the immunogen, but only weak IgG reactivity with the breast cancer cell line T47D was observed. Immunization of late-stage breast cancer patients with 33 or 105-mer MUC1 tandem repeat peptides coupled to KLH induced antibodies preferentially reactive with unglycosylated MUC1 peptides, although reactivity with Tn-MUC1 glycopeptides inversely proportional with density of Tn-glycosylation density was observed (Snijdewint et al. 2001). In a phase I study in non-small-cell lung cancer patients, vaccination with an unglycosylated MUC1 25-mer peptide in a liposomal formulation (BPL25) did not result in significant humoral immune responses (Palmer et al. 2001). In another phase I study enrolling 16 pancreatic cancer patients, vaccination with a 100-mer unglycosylated MUC1 peptide induced low levels of MUC1-specific antibodies in

some patients (Ramanathan et al. 2005). Whether these antibodies reacted with cancer-associated MUC1 is unknown.

Recognition of *O*-glycopeptide epitopes by the cellular immune system may further enhance immunity to the cancer-specific glycopeptide epitopes identified in this study. It has been shown that T cells can specifically recognize Tn-glycosylated peptides presented by MHC class II molecules without cross-response to other glycopeptides (Galli-Stampino et al. 1997). In one example, a 16-mer MUC1 tandem repeat peptide with Tn-glycosylation of Thr in the VTSA region was modified in the MHC anchor positions to optimize binding to the A(d) MHC class II molecule in the Balb/c mouse (Gad et al. 2003). Some of the anchor-enhanced glycopeptides were able to induce strong MUC1-specific T cell responses in Balb/c mice cross-reacting with the wildtype MUC1 glycopeptide. Furthermore, it has been shown that Tn-glycosylation of an anchor residue in an 8-mer MUC1 peptide can increase affinity to an MHC class I molecule by 100-fold compared with the corresponding unglycosylated peptide, and that GalNAc points into the central C anchor pocket (Apostolopoulos et al. 2003). Although *O*-glycosylation seems to be an unavoidable factor for driving a cancer-specific humoral response, *O*-glycosylation in the VTSA region has been reported to protect against the proteolysis required for processing and presentation by MHC class II molecules in antigen-presenting cells (Vlad et al. 2002). The latter may explain why we have, to date, been unable to detect evidence of cellular responses to the Tn/STn-MUC1 glycopeptide vaccines in MUC1 transgenic mice (unpublished observation).

Recent advances in the availability of recombinant glycosyltransferases have allowed chemoenzymatic synthesis of large glycopeptides with multiple *O*-glycans such as the 60-mer glycopeptides with up to 15 *O*-glycans. At present, this is not practical, using organic synthesis. Although, complete flexibility with respect to attachment sites of *O*-glycans cannot be accommodated, availability of an array of recombinant polypeptide GalNAc-transferases does allow a large degree of site-directed *O*-glycosylation (Defrees et al. 2006; Sorensen et al. 2006). In the present study, we extended previous chemoenzymatic synthesis of MUC1 glycoforms to include T, ST, and core 3. Similar to the finding with synthesis of STn (Sorensen et al. 2006), complete in vitro glycosylation of two and three *O*-glycans per MUC1 repeat is efficiently achieved; however, complete glycosylation of all five sites in all repeats was more difficult to obtain and in some cases impossible. Because glycoforms with 13–15 *O*-glycans are generally obtained, it is likely that the flanking repeats of the 60-mer peptide design are the cause of this, but further studies to address this will be needed. Chemoenzymatic synthesis of the extended MUC1 glycopeptides was performed in milligram scale with over 50% yields and it should be feasible to scale up the process for manufacturing. Alternatively, further studies to define the minimum glycopeptides required may be used for the design of a smaller hapten vaccine.

Interesting reactivity with the GlcNAcβ1-3GalNAcα1-*O*-Ser/Thr core 3 structures was observed with several antibodies. In particular, BW835, previously reported to define T-MUC1 (Hanisch et al. 1995), did not distinguish between the core 3 and T structures when presented on a fully glycosylated MUC1 peptide, and the results therefore suggest a broader specificity for this antibody. It should be noted that expression of GlcNAc-terminated core 3 structures is likely to be highly limited. Expression of the β3Gn-T6 enzyme synthesizing the core 3 *O*-glycan structure is limited to stomach, colon, and small intestine (Iwai et al. 2002). Moreover, masking of terminating GlcNAc residues as a result of galactosylation and sialylation would be expected.

In conclusion, we have demonstrated the existence of a novel, immunodominant, combined glycopeptide epitope in the MUC1 tandem repeat strictly dependent on a specific Tn/STn-glycosylation pattern with a remarkable cancer-specific expression pattern. The epitope is able to override tolerance in MUC1 transgenic mice, suggesting that it represents a prime target for vaccine design. We propose that a similar strategy may be applied with success to other cancer-associated mucins. Knowledge of the molecular nature and the availability of potent MAbs to this cancer-specific epitope are important for developing immunodiagnostic and therapeutic strategies based on MUC1.

Materials and methods

A MUC1 60-mer peptide $(VTSAPDTRPAPGSTAPPAHG)_{n=3}$ representing three tandem repeats was synthesized (by Cancer Research UK) as originally reported (Fontenot et al. 1993). For the immunization of mice, the peptide was completely Tn-glycosylated in vitro by concerted action of GalNAc-T2 and -T4 (see *Chemoenzymatic synthesis of glycopeptides*). For capture ELISA, an NH_2-terminal-biotinylated variant of the 60-mer peptide was in vitro-glycosylated to form 11 different glycoforms (Figure 1). Furthermore, as control peptide, a MUC2 33-mer peptide (PTTTPITTTTTVTPTPTPTGTQTPTTTPISTTC) corresponding to 1.4 tandem repeat (kindly provided by Dr P.O. Livingston) was Tn-glycosylated by GalNAc-T2 with an occupancy of approximately 12 out of 20 potential acceptor sites. Two valine-substituted, NH_2-terminal-biotinylated 25-mer MUC1 peptides, TAP25V9 (T^1APPAHGV**V**^{9}SAPDTR PAPGST^{21}APPA) and TAP25V21 (T^1APPAHGVT^{9}SAPD TRPAPGS**V**^{21}APPA), were synthesized and their glycosylation products with different polypeptide GalNAc-transferases characterized as previously described (Hassan et al. 2000). These peptides were enzymatically in vitro-glycosylated at Thr^1 and Thr^{21} or Thr^1 and Thr^9, respectively, using GalNAc-T11 (Schwientek et al. 2002) (Figure 1). Furthermore, eight different 21-mer MUC1 glycopeptides with either a single Tn-glycan (Tn-A1–Tn-A4) or a single T-glycan (T-A1–T-A4) based on the sequence AHGVTSAPDTRPAPGSTAPPA were chemically synthesized (Karsten et al. 1998) (Figure 1).

Chemoenzymatic synthesis of glycopeptides

All glycosylation reactions contained 0.4 mg peptide/mL and enzyme added as needed for terminal glycosylation. Synthesis of glycopeptides carrying the Tn-epitope (GalNAcα-*O*-Ser/Thr) was performed in reaction mixtures containing 25 mM sodium cacodylate buffer (pH 7.4), 10 mM $MnCl_2$, 0.25% Triton X-100, and 2 mM UDP-GalNAc. The density of glycosylation was controlled by the use of three different human polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-transferases) (EC 2.4.1.41).

GalNAc-T11 (Schwientek et al. 2002) was used to add two GalNAc residues per tandem repeat, GalNAc-T2 (White et al. 1995) to add three residues, and sequential use of GalNAc-T2 and -T4 (Bennett et al. 1998) to glycosylate all five potential *O*-glycosylation sites per tandem repeat. Sialylation of the Tn structure, rendering the STn structure, was performed with ST6GalNAc-I, a human α-*N*-acetylgalactosaminide α-2,6-sialyltransferase (Ikehara et al. 1999) (EC 2.4.99.3). The reaction mixture contained 50 mM MES (pH 6.5), 20 mM EDTA, 2 mM DTT, and 2 mM CMP-NeuAc. Core 1 synthesis was performed with dC1Gal-T1 (core 1 synthase), a glycoprotein-GalNAc 3-β-galactosyltransferase from *Drosophila melanogaster* (Ju et al. 2002) (EC 2.4.1.122). The reaction mixture contained 100 mM MES buffer (pH 6.5), 0.1% Triton X-100, 20 mM $MnCl_2$, and 2 mM UDP-Gal. Sialylation of the T structure, rendering the ST structure, was done in 100 mM MES (pH 6.5), 8 mM EDTA, 4 mM DTT, and 2 mM CMP-NeuAc by the use of ST3Gal-I, a human β-galactoside α-2,3-sialyltransferase (Shang et al. 1999) (EC 2.4.99.4). Core 3 synthesis was performed by using β3Gn-T6 (core 3 synthase), a human acetylgalactosaminyl-*O*-glycosyl-glycoprotein β-1,3-*N*-acetylglucosaminyltransferase (Iwai et al. 2002) (EC 2.4.1.147). The reaction mixture contained 50 mM sodium cacodylate buffer (pH 7.4), 0.4% CF-54, 10 mM $MnCl_2$, and 2 mM UDP-GlcNAc. Samples from enzyme reactions were diluted in 0.1% TFA to a final concentration of 40 ng/μL, and 0.5 μL was applied to the sample plate and mixed with 0.5 μL 2,5-DHB matrix (25 mg/mL in 30% acetonitrile). Samples were analyzed by MALDI-TOF MS on a Voyager-DE™ Pro Biospectrometry Workstation (Applied Biosystems, Stafford, TX) in positive linear or reflectron mode. Semipreparative reversed-phase high performance liquid chromatography (RP-HPLC) was performed on an 1100 series HPLC system mounted with a Zorbax 300SB-C18 column (Agilent Technologies, Waldbronn, Germany) eluting with a 40-min gradient from 0% to 90% acetonitrile in 0.1% TFA. Glycopeptides were lyophilized on a Labconco Lyph-lock 1L (Labconco Corporation, Kansas City, MO).

Conjugation of MUC1 60-mer glycopeptide for immunization

MUC1 peptide of 60-mer, carrying 15 GalNAc residues, was conjugated to Imject® mariculture keyhole limpet hemocyanin (mcKLH) (Pierce Biotechnology, Inc., Rockford, IL) using glutaraldehyde (Harlow and Lane 1988) in a molar ratio of glycopeptide:mcKLH 300:1. Excess glutaraldehyde was removed on PD-10 desalting columns (Amersham Biosciences, Uppsala, Sweden) eluting in PBS. Fractions were pooled on the basis of OD readings at 280 and 210 nm. The fractions corresponding to the elution time of the unconjugated peptides did not contain peptide according to OD reading at 210 nm. Furthermore, in ELISA, the rate of conjugation was estimated to be nearly complete by comparing reactivity of the conjugates and the corresponding unconjugated glycopeptides with MAbs directed to the glycopeptide or the glycan alone.

Production of recombinant MUC1 in CHO ldlD cells

CHO ldlD cells stably transfected with a soluble MUC1-murine IgG2a fusion construct containing 16 tandem repeats were cultured in the Iscove modified Dulbecco medium with 10% FCS and 600 μg/mL G418. Exploiting the deficiency of UDP-Gal/UDP-GalNAc 4-epimerase in these cells (Kingsley et al. 1986), culturing with 1 mM GalNAc yielded cells expressing soluble Tn-MUC1, whereas culturing with 1 mM GalNAc and 0.1 mM Gal yielded cells expressing soluble ST-MUC1. Glycoproteins (6 × His-tagged) were purified on Ni-NTA agarose (Qiagen, Hilden, Germany). Purified ST-MUC1 was treated with neuraminidase (0.2 U/mL in 50 mM sodium acetate buffer, pH 5.5) to render T-MUC1, followed by re-purification on Ni-NTA agarose for the removal of neuraminidase.

Generation of MAb 2D9

Similar to MAb 5E5, female Balb/c mice were immunized with 15Tn-MUC1 60-mer glycopeptide conjugated to KLH (Sorensen et al. 2006). Tail bleeds were collected 7 days after the third immunization and sera tested by ELISA, with the Tn-MUC2 glycopeptide serving as negative control, or by immunocytochemistry with CHO ldlD MUC1F cells expressing Tn-MUC1, ST-MUC1 (T-MUC1 after neuraminidase treatment) or unglycosylated MUC1, T47D (human ductal breast carcinoma), MCF7 (human breast carcinoma), and MTSV1−7 (human breast). Three days after the fourth immunization, spleen cells from one mouse were fused with NS1 myeloma cells (Kohler and Milstein 1975). Hybridomas specific to the antigens of interest were cloned by limiting dilution at least three times.

Other MAbs

Two control antibodies were raised in female Balb/c mice against purified soluble MUC1 from CHO ldlD MUC1 cells grown in GalNAc (MAb 5E10) or Gal and GalNAc, followed by neuraminidase treatment (MAb 1B9). Immunizations were performed by one subcutaneous injection of 40 μg/100 μL of immunogen emulsified in Freund's complete adjuvant, followed by two injections with Freund's incomplete adjuvant at 2−3 weeks intervals, and finally a boost without adjuvant. The two clones were selected by immunocytochemistry, as described in *Generation of MAb 2D9*, with different selection criteria. MAb 5E10 was selected because it reacted with all the tested MUC1-expressing cell lines independently of *O*-glycosylation and therefore potentially could serve as a universal anti-MUC1 MAb. MAb 1B9 was selected because it showed specificity for neuraminidase-treated cells presenting the T antigen.

Enzyme-linked immunosorbent assays

ELISA were performed using Nunc-Immuno MaxiSorp F96 plates (Nunc, Roskilde, Denmark). Unbiotinylated glycopeptides were serially diluted from an initial concentration of 2 μg/mL and coated 1 h at 37°C or overnight at 4°C in carbonate–bicarbonate buffer (pH 9.6). For capture ELISA, plates were coated 1 h at 37°C or overnight at 4°C with 1.5 μg/mL of streptavidin (Sigma-Aldrich, St Louis, MO) in carbonate–bicarbonate buffer (pH 9.6). Plates were blocked with SuperBlock Blocking Buffer (Pierce) for 1 h at room temperature. The streptavidin-coated plates were incubated with biotinylated glycopeptides serially diluted from an initial concentration of 2 μg/mL and incubated for 1 h at

37°C or overnight at 4°C. Subsequently, plates were incubated with MAbs for 2 h at room temperature or overnight at 4°C. 5E5, 2D9, 1B9, 5E10, and SM3 were used as undiluted culture supernatants, whereas MY.1E12 ascites were used 1:1000 and purified BW835 was used at 1 μg/mL. MY.1E12 was kindly provided by Dr T. Irimura, and BW835 by Drs F.-G. Hanisch and T. Schwientek. Sera from MUC1 transgenic mice immunized with Tn-MUC1 were serially diluted in 2% BSA in PBS from an initial dilution of 1:100 or 1:200. Bound antibodies were detected with HRP-conjugated polyclonal rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark). Plates were developed with TMB+ one-step substrate system (Dako), reactions stopped with 1 N H_2SO_4, and read at 450 nm.

Immunocytochemistry

Cell lines were fixed for 10 min in ice-cold acetone. Fixed cells were incubated overnight at 4°C with undiluted MAb supernatants, followed by incubation for 45 min at room temperature with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (Dako). Slides were mounted in glycerol-containing *p*-phenylenediamine and examined in a Zeiss fluorescence microscope (FluoresScience, Hallbergmoos, Germany).

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Conflict of interest statement

None declared.

Abbreviations

Core 1 (T), Galβ1-3GalNAcα1-*O*-Ser/Thr; Core 2, Galβ1-3[GlcNAcβ1-6]GalNAcα1-*O*-Ser/Thr; Core 3, GlcNAcβ1-3 GalNAcα1-*O*-Ser/Thr; ELISA, enzyme-linked immunosorbent assay; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HMFG, human milk fat globule; HPLC, high performance liquid chromatography; KLH, keyhole limpet hemocyanin; MAb, monoclonal antibody; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; NeuAc, sialic acid; RP-HPLC, reversed-phase HPLC; ST, NeuAcα2-3 Galβ1-3GalNAcα1-*O*-Ser/Thr; STn, NeuAcα2-6 GalNAcα1-*O*-Ser/Thr; T, Galβ1-3GalNAcα1-*O*-Ser/Thr; Tn, GalNAcα1-*O*-Ser/Thr.

References

Apostolopoulos V, Pietersz GA, Tsibanis A, Tsikkinis A, Drakaki H, Loveland BE, Piddlesden SJ, Plebanski M, Pouniotis DS, Alexis MN, et al. 2006. Pilot phase III immunotherapy study in early-stage breast cancer patients using oxidized mannan-MUC1 [ISRCTN71711835]. Breast Cancer Res. 8:R27.

Apostolopoulos V, Yuriev E, Ramsland PA, Halton J, Osinski C, Li W, Plebanski M, Paulsen H, McKenzie IF. 2003. A glycopeptide in complex with MHC class I uses the GalNAc residue as an anchor. Proc Natl Acad Sci USA. 100:15029–15034.

Backstrom M, Link T, Olson FJ, Karlsson H, Graham R, Picco G, Burchell J, Taylor-Papadimitriou J, Noll T, Hansson GC. 2003. Recombinant MUC1 mucin with a breast cancer-like *O*-glycosylation produced in large amounts in Chinese-hamster ovary cells. Biochem J. 376:677–686.

Bennett EP, Hassan H, Mandel U, Mirgorodskaya E, Roepstorff P, Burchell J, Taylor-Papadimitriou J, Hollingsworth MA, Merkx G, van Kessel AG, et al. 1998. Cloning of a human UDP-*N*-acetyl-alpha-D-Galactosamine:polypeptide *N*-acetylgalactosaminyltransferase that complements other GalNAc-transferases in complete *O*-glycosylation of the MUC1 tandem repeat. J Biol Chem. 273:30472–30481.

Beum PV, Singh J, Burdick M, Hollingsworth MA, Cheng PW. 1999. Expression of core 2 beta-1,6-*N*-acetylglucosaminyltransferase in a human pancreatic cancer cell line results in altered expression of MUC1 tumor-associated epitopes. J Biol Chem. 274:24641–24648.

Burchell J, Gendler S, Taylor-Papadimitriou J, Girling A, Lewis A, Millis R, Lamport D. 1987. Development and characterization of breast cancer reactive monoclonal antibodies directed to the core protein of the human milk mucin. Cancer Res. 47:5476–5482.

Burchell J, Taylor-Papadimitriou J, Boshell M, Gendler S, Duhig T. 1989. A short sequence, within the amino acid tandem repeat of a cancer-associated mucin, contains immunodominant epitopes. Int J Cancer. 44:691–696.

Correa I, Plunkett T, Coleman J, Galani E, Windmill E, Burchell JM, Taylor-Papdimitriou J. 2005. Responses of human T cells to peptides flanking the tandem repeat and overlapping the signal sequence of MUC1. Int J Cancer. 115:760–768.

Dai J, Allard WJ, Davis G, Yeung KK. 1998. Effect of desialylation on binding, affinity, and specificity of 56 monoclonal antibodies against MUC1 mucin. Tumour Biol. 19 Suppl 1:100–110.

Dalziel M, Whitehouse C, McFarlane I, Brockhausen I, Gschmeissner S, Schwientek T, Clausen H, Burchell JM, Taylor-Papadimitriou J. 2001. The relative activities of the C2GnT1 and ST3Gal-I glycosyltransferases determine *O*-glycan structure and expression of a tumor-associated epitope on MUC1. J Biol Chem. 276:11007–11015.

Defrees S, Wang ZG, Xing R, Scott AE, Wang J, Zopf D, Gouty DL, Sjoberg ER, Panneerselvam K, Brinkman-Van der Linden EC, et al. 2006. GlycoPegylation of recombinant therapeutic proteins produced in *E. coli*. Glycobiology. 16:833–843.

Dokurno P, Bates PA, Band HA, Stewart LM, Lally JM, Burchell JM, Taylor-Papadimitriou J, Snary D, Sternberg MJ, Freemont PS. 1998. Crystal structure at 1.95 A resolution of the breast tumour-specific antibody SM3 complexed with its peptide epitope reveals novel hypervariable loop recognition. J Mol Biol. 284:713–728.

Feuerer M, Beckhove P, Bai L, Solomayer EF, Bastert G, Diel IJ, Pedain C, Oberniedermayr M, Schirrmacher V, Umansky V. 2001. Therapy of human tumors in NOD/SCID mice with patient-derived reactivated memory T cells from bone marrow. Nat. Med. 7:452–458.

Fontenot JD, Tjandra N, Bu D, Ho C, Montelaro RC, Finn OJ. 1993. Biophysical characterization of one-, two-, and three-tandem repeats of human mucin (muc-1) protein core. Cancer Res. 53:5386–5394.

Gad M, Jensen T, Gagne R, Komba S, Daugaard S, Kroman N, Meldal M, Werdelin O. 2003. MUC1-derived glycopeptide libraries with improved MHC anchors are strong antigens and prime mouse T cells for proliferative responses to lysates of human breast cancer tissue. Eur J Immunol. 33: 1624–1632.

Galli-Stampino L, Meinjohanns E, Frische K, Meldal M, Jensen T, Werdelin O, Mouritsen S. 1997. T-cell recognition of tumor-associated carbohydrates: the nature of the glycan moiety plays a decisive role in determining glycopeptide immunogenicity. Cancer Res. 57:3214–3222.

Gilewski T, Adluri S, Ragupathi G, Zhang S, Yao TJ, Panageas K, Moynahan M, Houghton A, Norton L, Livingston PO. 2000. Vaccination of high-risk breast cancer patients with mucin-1 (MUC1) keyhole limpet hemocyanin conjugate plus QS-21. Clin Cancer Res. 6:1693–1701.

Grinstead JS, Koganty RR, Krantz MJ, Longenecker BM, Campbell AP. 2002. Effect of glycosylation on MUC1 humoral immune recognition: NMR studies of MUC1 glycopeptide-antibody interactions. Biochemistry. 41:9946–9961.

Grinstead JS, Schuman JT, Campbell AP. 2003. Epitope mapping of antigenic MUC1 peptides to breast cancer antibody fragment B27.29: a heteronuclear NMR study. Biochemistry. 42:14293–14305.

Hanisch FG, Stadie T, Bosslet K. 1995. Monoclonal antibody BW835 defines a site-specific Thomsen-Friedenreich disaccharide linked to threonine within the VTSA motif of MUC1 tandem repeats. Cancer Res. 55: 4036–4040.

Harlow E, Lane D. 1988. Antibodies: a laboratory manual. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.

Hassan H, Reis CA, Bennett EP, Mirgorodskaya E, Roepstorff P, Hollingsworth MA, Burchell J, Taylor-Papadimitriou J, Clausen H. 2000. The lectin domain of UDP-*N*-acetyl-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase-T4 directs its glycopeptide specificities. J Biol Chem. 275:38197–38205.

Hilkens J, Buijs F, Hilgers J, Hageman P, Calafat J, Sonnenberg A, van der Valk M. 1984. Monoclonal antibodies against human milk-fat globule membranes detecting differentiation antigens of the mammary gland and its tumors. Int J Cancer. 34:197–206.

Ikehara Y, Kojima N, Kurosawa N, Kudo T, Kono M, Nishihara S, Issiki S, Morozumi K, Itzkowitz S, Tsuda T, et al. 1999. Cloning and expression of a human gene encoding an *N*-acetylgalactosamine-alpha2,6-sialyltransferase (ST6GalNAc I): a candidate for synthesis of cancer-associated sialyl-Tn antigens. Glycobiology. 9:1213–1224.

Iwai T, Inaba N, Naundorf A, Zhang Y, Gotoh M, Iwasaki H, Kudo T Togayachi, A, Ishizuka Y, Nakanishi H, et al. 2002. Molecular cloning and characterization of a novel UDP-GlcNAc:GalNAc-peptide beta1,3-*N*-acetylglucosaminyltransferase (beta 3Gn-T6), an enzyme synthesizing the core 3 structure of *O*-glycans. J Biol Chem. 277: 12802–12809.

Ju T, Brewer K, D'Souza A, Cummings RD, Canfield WM. 2002. Cloning and expression of human core 1 beta1,3-galactosyltransferase. J Biol Chem. 277:178–186.

Karanikas V, Hwang LA, Pearson J, Ong CS, Apostolopoulos V, Vaughan H, Xing PX, Jamieson G, Pietersz G, Tait B, et al. 1997. Antibody and T cell responses of patients with adenocarcinoma immunized with mannan-MUC1 fusion protein. J Clin Invest. 100:2783–2792.

Karsten U, Diotel C, Klich G, Paulsen H, Goletz S, Muller S, Hanisch FG. 1998. Enhanced binding of antibodies to the DTR motif of MUC1 tandem repeat peptide is mediated by site-specific glycosylation. Cancer Res. 58:2541–2549.

Kingsley DM, Kozarsky KF, Hobbie L, Krieger M. 1986. Reversible defects in *O*-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. Cell. 44:749–759.

Kohler G, Milstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. J Immunol. 174:2453–2455.

Kufe D, Inghirami G, Abe M, Hayes D, Justi-Wheeler H, Schlom J. 1984. Differential reactivity of a novel monoclonal antibody (DF3) with human malignant versus benign breast tumors. Hybridoma. 3:223–232.

Lloyd KO, Burchell J, Kudryashov V, Yin BW, Taylor-Papadimitriou J. 1996. Comparison of *O*-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines. Demonstration of simpler and fewer glycan chains in tumor cells. J Biol Chem. 271:33325–33334.

Moller H, Serttas N, Paulsen H, Burchell JM, Taylor-Papadimitriou J. 2002. NMR-based determination of the binding epitope and conformational analysis of MUC-1 glycopeptides and peptides bound to the breast cancer-selective monoclonal antibody SM3. Eur J Biochem. 269: 1444–1455.

Mungul A, Cooper L, Brockhausen I, Ryder K, Mandel U, Clausen H, Rughetti A, Miles DW, Taylor-Papadimitriou J, Burchell JM. 2004. Sialylated core 1 based *O*-linked glycans enhance the growth rate of mammary carcinoma cells in MUC1 transgenic mice. Int J Oncol. 25: 937–943.

Nuti M, Turchi V, Rughetti A, Viacava P, Masci AM, Castagna M, Frati L. 1992. Characterization of monoclonal antibody 436 recognizing the Arg-Pro-Ala-Pro sequence of the polymorphic epithelial mucin (PEM) protein core in breast carcinoma cells. Int J Biol Markers. 7:71–79.

Palmer M, Parker J, Modi S, Butts C, Smylie M, Meikle A, Kehoe M, MacLean G, Longenecker M. 2001. Phase I study of the BLP25 (MUC1 peptide) liposomal vaccine for active specific immunotherapy in stage IIIB/IV non-small-cell lung cancer. Clin Lung Cancer. 3:49–57.

Petrarca C, Rughetti A, Rahimi H, D'Agostini F, Turchi V, Apollonj GC, Scambia G, Frati L, Nuti M. 1996. Human antibodies against the polymorphic epithelial mucin in ovarian cancer patients recognise a novel sequence in the tandem repeat region. Eur J Cancer. 32A: 2155–2163.

Ramanathan RK, Lee KM, McKolanis J, Hitbold E, Schraut W, Moser AJ, Warnick E, Whiteside T, Osborne J, Kim H, et al. 2005. Phase I study of a MUC1 vaccine composed of different doses of MUC1 peptide with SB-AS2 adjuvant in resected and locally advanced pancreatic cancer. Cancer Immunol Immunother. 54:254–264.

Reis CA, Hassan H, Bennett EP, Clausen H. 1998. Characterization of a panel of monoclonal antibodies using GalNAc glycosylated peptides and recombinant MUC1. Tumour Biol. 19 Suppl 1:127–133.

Rughetti A, Pellicciotta I, Biffoni M, Backstrom M, Link T, Bennet EP, Clausen H, Noll T, Hansson GC, Burchell JM, et al. 2005. Recombinant tumor-associated MUC1 glycoprotein impairs the differentiation and function of dendritic cells. J Immunol. 174: 7764–7772.

Rughetti A, Turchi V, Ghetti CA, Scambia G, Panici PB, Roncucci G, Mancuso S, Frati L, Nuti M. 1993. Human B-cell immune response to the polymorphic epithelial mucin. Cancer Res. 53:2457–2459.

Ryuko K, Schol DJ, Snijdewint FG, von Mensdorff-Pouilly S, Poort-Keesom RJ, Karuntu-Wanamarta YA, Verstraeten RA, Miyazaki K, Kenemans P, Hilgers J. 2000. Characterization of a new MUC1 monoclonal antibody (VU-2-G7) directed to the glycosylated PDTR sequence of MUC1. Tumour Biol. 21:197–210.

Samuel J, Longenecker BM. 1995. Development of active specific immunotheropeutic agents based on cancer-associated mucins. Pharm Biotechnol. 6:875–890.

Schwientek T, Bennett EP, Flores C, Thacker J, Hollmann M, Reis CA, Behrens J, Mandel U, Keck B, Schafer MA, et al. 2002. Functional conservation of subfamilies of putative UDP-*N*-acetylgalactosamine:polypeptide *N*-acetylgalactosaminyltransferases in *Drosophila, Caenorhabditis elegans*, and mammals. One subfamily composed of l(2)35Aa is essential in *Drosophila*. J Biol Chem. 277:22623–22638.

Shang J, Qiu R, Wang J, Liu J, Zhou R, Ding H, Yang S, Zhang S, Jin C. 1999. Molecular cloning and expression of Galbeta1,3GalNAc alpha2, 3-sialyltransferase from human fetal liver. Eur J Biochem. 265: 580–588.

Snijdewint FG, von Mensdorff-Pouilly S, Karuntu-Wanamarta AH, Verstraeten AA, Livingston PO, Hilgers J, Kenemans P. 2001. Antibody-dependent cell-mediated cytotoxicity can be induced by MUC1 peptide vaccination of breast cancer patients. Int J Cancer. 93:97–106.

Sorensen AL, Reis CA, Tarp MA, Mandel U, Ramachandran K, Sankaranarayanan V, Schwientek T, Graham R, Taylor-Papadimitriou J, Hollingsworth MA, et al. 2006. Chemoenzymatically synthesized multimeric Tn/STn MUC1 glycopeptides elicit cancer-specific anti-MUC1 antibody responses and override tolerance. Glycobiology. 16: 96–107.

Spencer DI, Missailidis S, Denton G, Murray A, Brady K, Matteis CI, Searle MS, Tendler SJ, Price MR. 1999. Structure/activity studies of the anti-MUC1 monoclonal antibody C595 and synthetic MUC1 mucin-core-related peptides and glycopeptides. Biospectroscopy. 5:79–91.

Springer GF. 1984. T and Tn, general carcinoma autoantigens. Science. 224: 1198–1206.

Takeuchi H, Kato K, da-Nagai K, Hanisch FG, Clausen H, Irimura T. 2002. The epitope recognized by the unique anti-MUC1 monoclonal antibody MY.1E12 involves sialyl alpha 2-3galactosyl beta 1-3*N*-acetylgalactosaminide linked to a distinct threonine residue in the MUC1 tandem repeat. J Immunol Methods. 270:199–209.

Taylor-Papadimitriou J, Epenetos AA. 1994. Exploiting altered glycosylation patterns in cancer: progress and challenges in diagnosis and therapy. Trends Biotechnol. 12:227–233.

Taylor-Papadimitriou J, Peterson JA, Arklie J, Burchell J, Ceriani RL, Bodmer WF. 1981. Monoclonal antibodies to epithelium-specific components of the human milk fat globule membrane: production and reaction with cells in culture. Int J Cancer. 28:17–21.

Vlad AM, Muller S, Cudic M, Paulsen H, Otvos L Jr, Hanisch FG, Finn OJ. 2002. Complex carbohydrates are not removed during processing of glycoproteins by dendritic cells: processing of tumor antigen MUC1 glycopeptides for presentation to major histocompatibility complex class II-restricted T cells. J Exp Med. 196:1435–1446.

von Mensdorff-Pouilly S, Petrakou E, Kenemans P, van UK, Verstraeten AA, Snijdewint FG, van Kamp GJ, Schol DJ, Reis CA, Price MR, et al. 2000a. Reactivity of natural and induced human antibodies to MUC1 mucin with MUC1 peptides and n-acetylgalactosamine (GalNAc) peptides. Int J Cancer. 86:702–712.

von Mensdorff-Pouilly S, Verstraeten AA, Kenemans P, Snijdewint FG, Kok A, van Kamp GJ, Paul MA, Van Diest PJ, Meijer S, Hilgers J. 2000b. Survival in early breast cancer patients is favorably influenced by a natural humoral immune response to polymorphic epithelial mucin. J Clin Oncol. 18:574–583.

Wandall HH, Hassan H, Mirgorodskaya E, Kristensen AK, Roepstorff P, Bennett EP, Nielsen PA, Hollingsworth MA, Burchell J, Taylor-Papadimitriou J, et al. 1997. Substrate specificities of three members of the human UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase family, GalNAc-T1, -T2, and -T3. J Biol Chem. 272:23503–23514.

White T, Bennett EP, Takio K, Sorensen T, Bonding N, Clausen H. 1995. Purification and cDNA cloning of a human UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase. J Biol Chem. 270:24156–24165.

Xing PX, Apostolopoulos V, Pietersz G, McKenzie IF. 2001. Anti-mucin monoclonal antibodies. Front Biosci. 6:D1284–D1295.

Xing PX, Prenzoska J, Quelch K, McKenzie IF. 1992. Second generation anti-MUC1 peptide monoclonal antibodies. Cancer Res. 52: 2310–2317.

Yamamoto M, Bhavanandan VP, Nakamori S, Irimura T. 1996. A novel monoclonal antibody specific for sialylated MUC1 mucin. Jpn J Cancer Res. 87:488–496.