Glycobiology vol. 14 no. 8 pp. 681–692, 2004
doi:10.1093/glycob/cwh090
Advance Access publication on April 28, 2004

Binding patterns of DTR-specific antibodies reveal a glycosylation-conditioned tumor-specific epitope of the epithelial mucin (MUC1)

Uwe Karsten[1,2,3], Nida Serttas[4], Hans Paulsen[4], Antje Danielczyk[5], and Steffen Goletz[2,5]

[2]NEMOD GmbH & Co. KG, Robert-Rössle-Str. 10, D-13125 Berlin, Germany; [3]Max Delbrück Centre for Molecular Medicine, D-13125 Berlin-Buch, Germany; [4]Institute of Organic Chemistry, University of Hamburg, D-20146 Hamburg, Germany; and [5]Glycotope GmbH, D-13125 Berlin-Buch, Germany

Received on February 6, 2004; revised on April 20, 2004; accepted on April 20, 2004

Glycosylation determines essential biological functions of epithelial mucins in health and disease. We report on the influence of glycosylation of the immunodominant DTR motif of MUC1 on its antigenicity. Sets of novel glycopeptides were synthesized that enabled us to examine sole and combined effects of peptide length (number of repeats) and *O*-glycosylation with GalNAc at the DTR motif on the binding patterns of 22 monoclonal antibodies recognizing this motif. In case of unglycosylated peptides almost all antibodies bound better to multiple MUC1 tandem repeats. Glycosylation at the DTR led to enhanced binding in 11 cases, whereas 10 antibodies were not influenced in binding, and one was inhibited. In nine of the former cases both length and DTR glycosylation were additive in their influence on antibody binding, suggesting that both effects are different. Improved binding to the glycosylated DTR motif was exclusively found with antibodies generated against tumor-derived MUC1. Based on these data a tumor-specific MUC1 epitope is defined comprising the ...PDTRP... sequence in a particular conformation essentially determined by *O*-glycosylation at its threonine with either GalNAcα1 or a related short glycan. The results can find application in the field of MUC1-based immunotherapy.

Key words: conformation/glycosylation/MUC1 antibodies/tumor epitope/tumor vaccine

Introduction

MUC1 is a highly *O*-glycosylated transmembrane glycoprotein (mucin) typically but not exclusively expressed on glandular epithelia and on epithelial tumors. It is an established serum tumor marker, especially in breast cancer patients (Hayes *et al*., 1986; Safi *et al*., 1991), and has in recent years attracted increasing attention as a potential target of tumor immunotherapies (Taylor-Papadimitriou *et al*., 1999; Finn *et al*., 1995). Whereas the case for the former is clear-cut, the rationale for the latter is not, especially with respect to the selection criteria for antibodies suitable as targeting vehicles and the design of efficient vaccines.

In the mid-1990s a plethora of monoclonal anti-MUC1 antibodies became available that required comparison and standardization. Thus the ISOBM TD-4 International Workshop on Monoclonal Antibodies against MUC1 in 1996 became a landmark in the field. Fifty-six supposedly MUC1-specific monoclonal antibodies (mAbs) were compared and their epitopes mapped (Rye and Price, 1998).

At that time it was well established that tumor MUC1—similar to other membrane glycoproteins—differs from normal MUC1 by modified (essentially truncated) glycan side chains, which results in a better accessibility of peptide epitopes (Brockhausen, 1999; Burchell *et al*., 2001; Lloyd *et al*., 1996; Taylor-Papadimitriou *et al*., 1999). In addition, it was assumed that the immunodominant DTR motif was generally not glycosylated (Finn *et al*., 1995). Thus the tumor specificity of anti-MUC1 mabs was interpreted solely in terms of better access to peptide epitopes due to reduced interference by glycans located in the vicinity of the epitope. This picture changed when we found that the DTR sequence is actually glycosylated (Müller *et al*., 1997) and that short glycans typical for tumor MUC1 (GalNAcα1- or Galβ1-3GalNAcα1-) at this site may even improve antibody binding to the DTR epitope (Karsten *et al*., 1998). Our hypothesis was that glycosylation at this site might induce a knoblike structure similar to that described for repetitive non-glycosylated tandem repeat peptides (Fontenot *et al*., 1995). Employing a large panel of monoclonal anti-MUC1 antibodies and three sets of specially devised antigenic structures, among them a unique set of novel synthetic glycopeptides, we continued our studies with the aim of a more refined epitope characterization. As a result, we were able to (1) dissect the glycosylation effect from the length effect, (2) describe subgroups of anti-MUC1 antibodies recognizing the DTR motif, and (3) define a novel tumor-specific MUC1 epitope.

Results

Length dependency

We first reexamined the binding patterns of the antibody panel listed in Table I toward nonglycosylated MUC1 peptides of increased length (oligomerization of 20-amino-acid-long tandem repeats). For this purpose, we determined the absorbance (A) increment of antibody binding occurring during prolongation from one up to six tandem repeat lengths. For the purpose of data reduction we included in Table II only the absorbance ratios $A_{100\text{-mer}}$:$A_{20\text{-mer}}$, and

[1]To whom correspondence should be addressed; e-mail: uwe.karsten@nemod.com

Table I. Antibodies examined in this study[a]

Clone	TD-4 #	Isotype	Immunogen	Epitope[b]
Ma552	122	G1	ZR75–1 cells	GVTSAPDTRPAP
BC3	123	M	HMFG[c]	APDTR
VU-3C6	125	G1	ZR75–1 cells	GVTSAPDTRPAP
VU-12E1	126	G1	ZR75–1 cells	PDTRPAP
MF06	129	G1	Ovarian cyst fluid	DTRPAP
VA1	131	G1	100-mer peptide	TRPAP
BCP8	133	G2b	VNTR[d] peptide	PDTRPA
BC2	138	G1	HMFG	APDTR
B27.29	139	G1	MUC1 from ascites	PDTRPAP
VU-3D1	140	G1	ZR75–1 cells	SAPDTRPAP
VU-4H5	144	G1	60-mer peptide	APDTRPAP
BCP9	147	G1	VNTR peptide	PAPGSTAP
MF11	149	G1	Ovarian cyst fluid	PPAH
BC4E549	153	G1	T-47D membranes	TSAPDTRPAP
VU-11E2	156	G1	ZR75–1 cells	TSAPDTRP
E29	159	G2a	HMFG	APDTRP
214D4	162	G1	VNTR peptide	PDTR
SM3	165	G1	HMFG (deglyc.)[e]	APDTRP
HMFG-1	169	G1	HMFG	APDTR
VA2	170	G1	100-mer	APDTRPA
b-12	171	G1	Cell line mixture	PDTRPAP
C595	172	G3	Urinary MUC1	TRPAP
Mc5	175	G1	HMFG	DTRPAP
A76-A/C7	177	G1	T-47D cells	APDTRPAP

[a]Data taken from Price *et al.* (1998).
[b]Nominal peptide epitopes as agreed by the participants of the Workshop.
[c]Human milk fat globule membranes.
[d]Variable number of tandem repeats; expression used for tandem repeat sequences.
[e]Partially deglycosylated (Burchell *et al.*, 1987).

classified these values into groups of length dependency as follows. LD-1: absolutely dependent (A of the 20-mer < 0.1 irrespective of their A_{100}:A_{20} ratio); LD-2: strongly dependent (ratio > 3.0); LD-3: weakly influenced (ratio 1.5–2.9); LI: independent (ratio < 1.5). Almost all antibodies showed increased binding toward the longer peptides, but this increase differed considerably among individual antibodies. A subgroup was almost nonreactive with the single repeat but revealed significant and increasing binding with increased numbers of repeats.

Two control antibodies recognizing non-DTR epitopes (BCP9, MF11) were found weakly influenced by peptide length (LD-3) in their binding. MF11 was not binding to the 20-mer, obviously because its epitope was too close to the end of the peptide.

Glycosylation dependency

The unexpected observation made by us in 1997 that many antibodies specific for the immunodominant DTR region of MUC1 bound better to the glycosylated than to the unglycosylated epitope (Karsten *et al.*, 1998) was reexamined in this study with a specially devised 30-mer peptide/glycopeptide pair of test antigens with the DTR motif situated in the middle of the peptide, and its threonine *O*-glycosylated with GalNAcα in the glycopeptide variant. Absorbance ratios $A_{glycosylated}$:$A_{unglycosylated}$ were calculated for each antibody and are presented in Table II. These ratios were taken from one experiment but confirmed in repeated experiments of the same kind and also in different experiments employing the same but N-terminally biotinylated peptide and glycopeptide, respectively. No qualitative differences were observed between direct coating to the polystyrene surface and coating via biotin/streptavidin, confirming that the glycosylation effect was not due to a different degree of binding of glycopeptides as compared to their unglycosylated counterparts on the solid phase. Differences in absorbance seen between individual experiments did not change in any case the classification of the antibodies, which was done as follows. GD-1: binding strongly dependent on DTR glycosylation (ratio > 6);

Table II. Summary of data for length and glycosylation dependencies of antibody binding

	Length dependency		Glycosylation dependency	
Clone	Ratio[a]	Group[b]	Ratio[c]	Group[d]
Ma552	2.1	LD-1	2.1	GD-2
BC3	1.8	LD-3	0.9	GI
VU-3C6	3.1	LD-1	5.4	GD-2
VU-12E1	3.7	LD-2	4.4	GD-2
MF06	1.8	LD-3	2.7	GD-2
VA1	2.2	LD-3	0.8	GI
BCP8	1.9	LD-3	1.0	GI
BC2	3.6	LD-2	1.0	GI
B27.29	2.7	LD-3	1.9	GD-2
VU-3D1	2.9	LD-3	4.2	GD-2
VU-4H5	5.8	LD-2	0.0	*i*GD
BCP9	1.6	LD-3	1.1	GI
MF11	4.5[e]	LD-3[f]	1.1	GI
BC4E549	4.5	LD-2	4.3	GD-2
VU-11E2	11.8	LD-1	16.3	GD-1
E29	2.5	LD-3	1.2	GI
214D4	1.6	LD-3	1.0	GI
SM3	2.1	LD-3	1.4	GD-2
HMFG-1	4.7	LD-2	1.0	GI
VA2	3.9	LD-2	1.2	GI
b-12	2.2	LD-3	4.5	GD-2
C595	1.1	LI	1.0	GI
Mc5	5.2	LD-2	1.0	GI
A76-A/C7	12.7	LD-1	18.0	GD-1

[a]Absorbance ratio $A_{100\text{-mer}}$:$A_{20\text{-mer}}$.
[b]LD-1: absolutely dependent (A of the 20-mer < 0.1 irrespective of their A_{100}:A_{20} ratio); LD-2: strongly dependent (ratio > 3.0); LD-3: weakly dependent (ratio 1.5–2.9); LI: independent (ratio < 1.5). These data were obtained with compounds of series I (see *Materials and methods*).
[c]Absorbance ratio $A_{glycosylated}$:$A_{unglycosylated\ peptide}$.
[d]GD-1: strongly dependent (ratio > 6); GD-2: moderately dependent (ratio 1.3–5.9); GI: independent (ratio 0.8–1.2); *i*GD: inversely influenced (binding inhibited, ratio < 0.8). These data were obtained with compounds of series II (see *Materials and methods*).
[e]This quotient is invalid due to the zero reactivity with the 20-mer (the epitope of this mAb is too close to the end of the 20-mer; compare Table VI and Figure 3B).
[f]Classified as LD-3 due to a slight A increment from the 60-mer to the 120-mer.

GD-2: moderately influenced (ratio 1.3–5.9); GI: independent of glycosylation (ratio 0.8–1.2); *i*GD: inversely influenced (binding inhibited) by glycosylation (ratio < 0.8).

The results showed that about half of the antibodies were glycosylation-influenced, that is, revealed better binding to the glycosylated epitope. Only in one case (VU-4H5) was inhibition of antibody binding observed after glycosylation of the epitope with GalNAc.

Both control antibodies recognizing non-DTR epitopes (BCP9, MF11) were found not influenced by DTR glycosylation in their binding, as could have been anticipated.

Combined glycosylation and length effects

Both the length effect and the glycosylation effect suggest a modification of the conformation of the DTR region as the reason for improved antibody binding, which for the length effect has indeed been demonstrated (Fontenot *et al.*, 1993). Therefore, we were interested to examine whether increasing peptide length and glycosylation at the DTR lead to the same assumed conformation as indicated by an identical maximum absorbance or whether both effects are additive and can be distinguished. This question could be resolved with a novel series of glycopeptides (*O*-glycosylated with GalNAc at the threonine of each DTR motif) of different length (one to five tandem repeats); see Table III.

The results demonstrate that the length effect and the effect of DTR glycosylation on antibody binding can be clearly distinguished. Figure 1 presents examples of binding patterns in response to oligomerization of MUC1 glycopeptides found among glycosylation-dependent antibodies. As can be seen, the increment in absorbance varies from strong to weak, and there are also cases where no additional length effect can be observed (Figure 1D). The frequencies of these four types of reactivity can be taken from Table IV (last column). Nine out of the 11 antibodies of this group revealed an additive influence of glycosylation and length, clearly indicating that both effects are different.

Antibodies not influenced by DTR glycosylation reproduce in most cases with glycosylated peptides the length dependency patterns seen with naked peptides. An effect of increased length was seen in 4 out of 11 cases (Table V), with only one mAb (HMFG-1) showing strong influence (see Figure 4, later). An exception was mAb Mc5, which revealed different binding patterns to glycosylated versus unglycosylated peptides with respect to length dependency. This case also provides evidence that the length effect is not an artifact of coating (e.g., due to an improved polystyrene binding of longer glycopeptides); the same conclusion can be drawn from the length-independent binding of mAb C595 with naked peptides. Weakly length-influenced mAbs (LD-3) did not show any length increment with oligomeric glycosylated peptides (Figure 2A–C). An interesting case is mAb VU-4H5 (Figure 2D), which was inhibited by DTR glycosylation but clearly responded to increased peptide length.

Binding patterns of the two antibodies selected as internal controls are shown in Table VI and Figure 3. As expected, their binding was independent of glycosylation, and the influence of peptide length, if any, was marginal. In case of mAb MF11 (Figure 3B), the epitope was obviously not sufficiently exposed in the 20-mer.

Periodate oxidation

Periodate treatment according to Woodward *et al.* (1985) is a valuable tool to confirm carbohydrate involvement in immunological recognition. This treatment cleaves sugar rings between vicinal OH groups and renders the structure unrecognizable for a specific antibody. It was therefore of interest to examine to what extent the observed effects were

Table III. Novel synthetic glycopeptides used in this study (series III)

Denomination	Structure	Formula	M_r calculated	M_r measured
TR1a	AHGVTSAPDT(GalNAc)RPAPGSTAPPA	$C_{91}H_{145}N_{27}O_{34}$	2161.34	2160.69
TR2a	A[HGVTSAPDT(GalNAc)RPAPGSTAPPA]$_2$	$C_{179}H_{283}N_{53}O_{66}$	4233.59	4233.66
TR3a	A[HGVTSAPDT(GalNAc)RPAPGSTAPPA]$_3$	$C_{267}H_{421}N_{79}O_{98}$	6305.84	6306.71
TR4a	A[HGVTSAPDT(GalNAc)RPAPGSTAPPA]$_4$	$C_{355}H_{559}N_{105}O_{130}$	8378.08	8377.61
TR5a	A[HGVTSAPDT(GalNAc)RPAPGSTAPPA]$_5$	$C_{443}H_{697}N_{131}O_{162}$	10,450.33	(10,450.67)

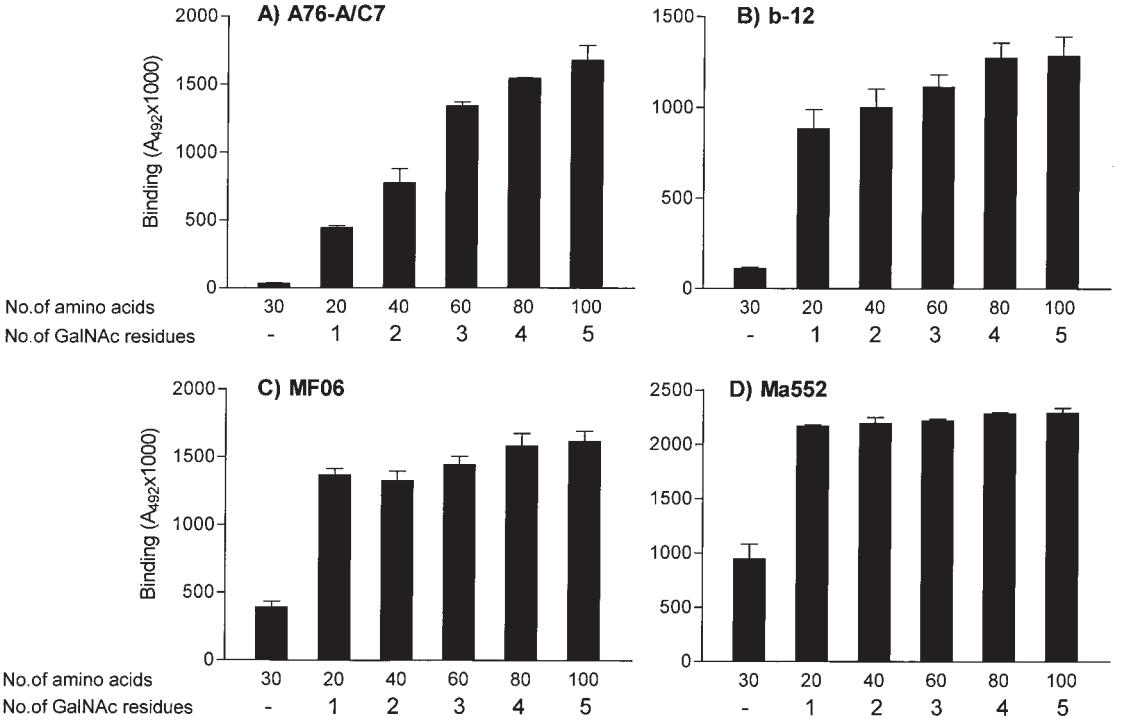

Fig. 1. Binding patterns of antibodies generated against tumor MUC1 to an unglycosylated VNTR peptide (first column) and to glycosylated VNTR peptides of different length (one to five repeats). All bind better to DTR-glycosylated peptides. A subgroup reveals an additional binding increment with increasing number of repeats (A strong, B moderate, C weak).

actually carbohydrate-induced, and if so, whether they were reversible.

The results shown in Figure 4 confirmed that the binding of mAb A76-A/C7 is glycosylation-conditioned (Figure 4A). However, the length effect partially compensates for the decrease in binding caused by periodate oxidation. This confirms the conclusion that both effects may overlap but are not identical. A different binding pattern was provided by mAb HMFG-1, whose binding is strongly influenced by the number of tandem repeats but not by DTR glycosylation (Figure 4B). An antibody classified as being neither glycosylation- nor length-dependent, E29, revealed a binding pattern as expected in this case and was not influenced by periodate oxidation of the glycopeptide in its binding (Figure 4C). This is also proof that the peptide was not damaged under these conditions. Interestingly, binding of antibody VU-4H5, which is suppressed by DTR glycosylation, was not restored after periodate oxidation but increased moderately with increased peptide length (Figure 4D). The unique behavior of mAb VU-4H5 suggests steric hindrance of antibody binding by the glycan even after oxidative ring cleavage and again demonstrates the length effect as an independent phenomenon.

Search for possible correlations

The fact that about half of the antibodies bound better to the glycosylated epitope initiated a search for possible correlations to other known properties of the antibodies, such as length dependency, isotype, epitope characteristics, immunogen, or the efficiency to detect carcinoma-specific MUC1. Except length and DTR-glycosylation influence, which were based on our own data (Table II), the other parameters were taken from the reports of the 1996 workshop (Rye and Price, 1998). In detail, isotypes, peptide

Table IV. Binding patterns of MUC1 antibodies: glycosylation-dependent antibodies correlate with tumor MUC1 as immunogen

Clone	Epitope[a]	Immunogen[a]	Efficiency points[b]	Series I Length effect[c]				Series II Glycosylation effect[c]				Series III Combined effects[d]
				LD-1	LD-2	LD-3	LI	GD-1	GD-2	GI	*i*GD	
VU-11E2	TSA**PDTRP**	ZR75–1 cells	<1	+				+				strong
A76-A/C7	A**PDTRP**AP	T-47D cells	70	+				+				strong
VU-3C6	GVTSA**PDTRP**AP	ZR75–1 cells	<1	+					+			weak
Ma552	GVTSA**PDTRP**AP	ZR75–1 cells	37	+					+			none
VU-12E1	**PDTRP**AP	ZR75–1 cells	4		+				+			moderate
BC4E549	TSA**PDTRP**AP	T-47D membr	<1		+				+			moderate
VU-3D1	SA**PDTRP**AP	ZR75–1 cells	<1			+			+			moderate
b-12	**PDTRP**AP	Cell line mix	144			+			+			moderate
B27.29	**PDTRP**AP[e]	MUC1 from asc	89			+			+			moderate
MF06	**DTRP**AP	Ovarian cyst fl	91			+			+			weak
SM3	A**PDTRP**	Deglyc. HMFG[f]	<1			+			+			none

[a]Taken from Price *et al.* (1998, tables 5 and 2, respectively). Abbreviations: membr = membranes; fl = fluid; asc = ascites.
[b]Taken from Norum *et al.* (1998, table 3). Efficiency points represent the ability to catch and present serum MUC1 from cancer patients in immunoradiometric assays.
[c]Data taken from Table III.
[d]Increment of absorbance with oligomerization of glycosylated MUC1 peptides.
[e]A second binding site has been recently described (Grinstead *et al.*, 2002).
[f]Partially deglycosylated human milk fat globule membranes (Burchell *et al.*, 1987), see *Results*.

Table V. Binding patterns of MUC1 antibodies: glycosylation-independent antibodies correlate with nontumor MUC1 as immunogen

				Series I Length effect				Series II Glycosylation effect				
Clone	Epitope[a]	Immunogen[a]	Efficiency points[b]	LD-1	LD-2	LD-3	LI	GD-1	GD-2	GI	*i*GD	Series III Length increment[c]
HMFG-1	AP**DTR**	HMFG	<1		+					+		strong
VU-4H5	AP**DTR**PAP	60-mer	3		+						+	moderate
BC2	AP**DTR**	HMFG	<1		+					+		weak
VA2	AP**DTR**PA	100-mer	<1		+					+		weak
Mc5	**DTR**PAP	HMFG	19		+					+		none
BC3	AP**DTR**	HMFG	53			+				+		none
VA1	**TR**PAP	100-mer	ND[d]			+				+		none
BCP8	P**DTR**PA	VNTR peptide	<1			+				+		none
E29	AP**DTR**P	HMFG	<1			+				+		none
214D4	P**DTR**	VNTR peptide	124			+				+		none
C595	**TR**PAP	Urinary MUC1	19				+			+		none

[a]Taken from Price *et al.* (1998, tables 5 and 2, respectively). Abbreviations: HMFG = human milk fat globule membranes; VNTR = (variable number of) tandem repeats.
[b]Taken from Norum *et al.* (1998, table 3). Efficiency points represent the ability to catch and present serum MUC1 from cancer patients in immunoradiometric assays.
[c]Increment of absorbance with oligomerization of glycosylated MUC1 peptides.
[d]ND = not done.

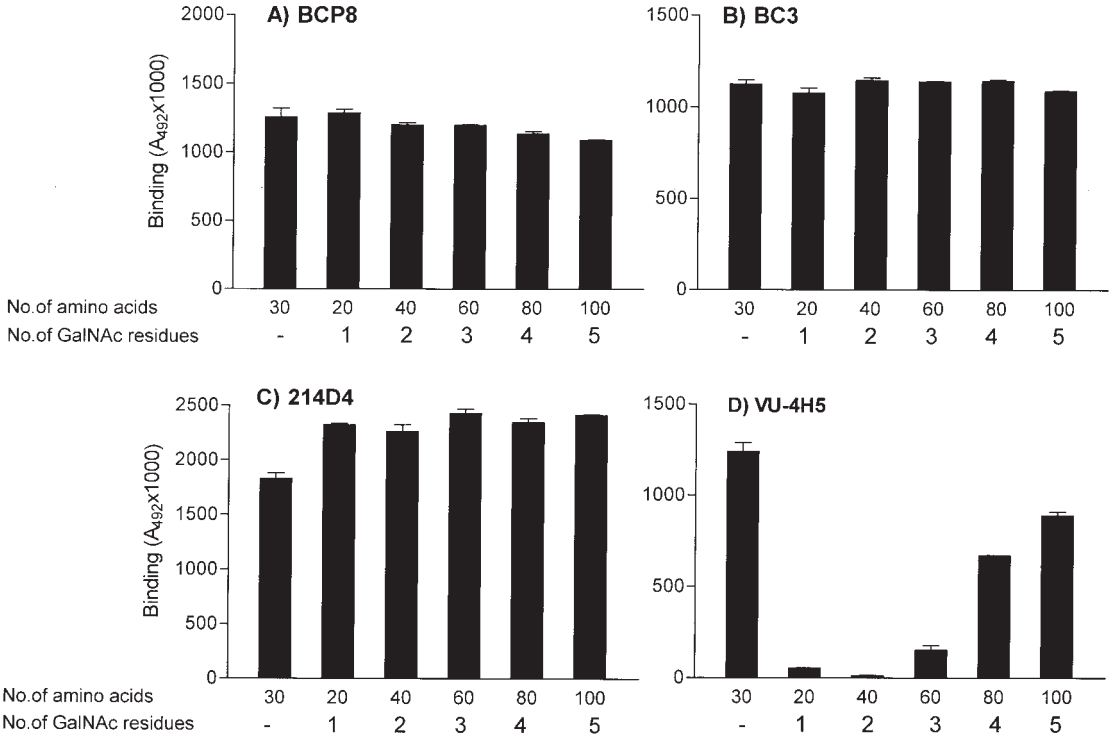

Fig. 2. Binding patterns of antibodies generated against nontumor MUC1 to an unglycosylated VNTR peptide (first column) and to glycosylated VNTR peptides of different length (one to five repeats). No significant increase of binding to DTR-glycosylated peptides is observed. One antibody (**D**) is not binding to glycosylated DTR but reveals a binding increment with increasing number of repeats.

Table VI. Binding patterns of MUC1 antibodies: control antibodies recognizing non-DTR epitopes

				Series I Length effect				Series II Glycosylation effect				
Clone	Epitope[a]	Immunogen[a]	Efficiency points[b]	LD-1	LD-2	LD-3	LI	GD-1	GD-2	GI	*i*GD	Series III Length increment[c]
BCP9	PAPGSTAP PPAH	VNTR peptide	<1				+				+	none
MF11		Ovarian cyst fluid	23				+				+	none

[a]Taken from Price *et al.* (1998, tables 5 and 2, respectively). Abbreviations: VNTR = (variable number of) tandem repeats.
[b]Taken from Norum *et al.* (1998, table 3). Efficiency points represent the ability to catch and present serum MUC1 from cancer patients in immunoradiometric assays.
[c]Increment of absorbance with oligomerization of glycosylated MUC1 peptides.

sequences agreed on as epitopes and immunogens were taken from the Summary Report (Price *et al.*, 1998), whereas the efficiency data were from Table 3 in Norum *et al.* (1998). The latter were arbitrary points based on the efficiency with which solid phase-coated antibodies presented shed tumor MUC1 (from a pool of patients' sera) for a given tracer antibody in immunoradiometric sandwich assays.

Among the examined parameters, the strongest correlation was found between glycosylation dependency and the type of immunogen used for generating the antibody (Tables IV and V). In fact, among the antibodies included in the study, all mAbs generated with immunogens from human tumor sources (e.g., breast cancer cells or MUC1 prepared from tumor ascites) bound better to the DTR-glycosylated peptide than to the naked peptide. The extent of this phenomenon varied from strong to weak. On the other hand, almost all mAbs generated with immunogens from nontumor sources (e.g., naked peptides or milk fat globule membranes) were glycosylation-independent. The only exception was SM3, which was generated with partially deglycosylated HMFG as immunogen, and was slightly glycosylation-influenced. It should be mentioned, however, that this preparation was treated with anhydrous hydrogen fluoride (Burchell *et al.*, 1987), which, under mild

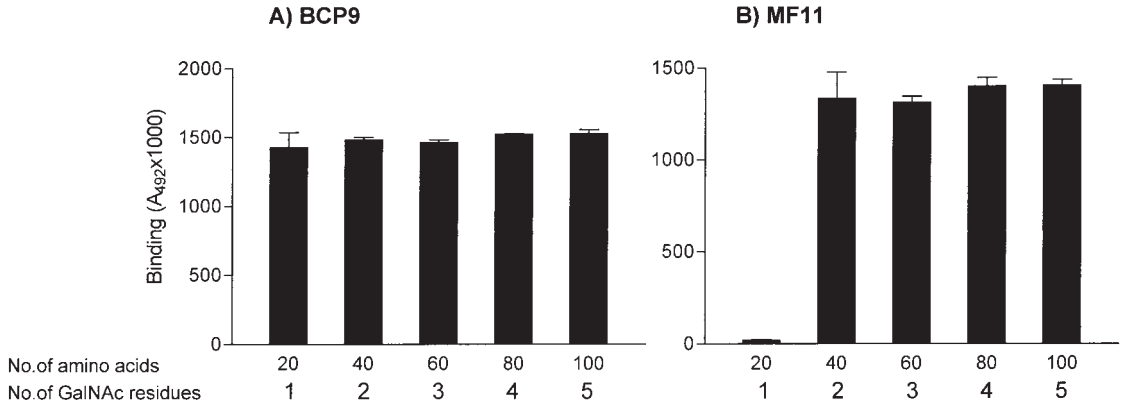

Fig. 3. Binding patterns of two control antibodies recognizing MUC1 epitopes different from DTR to glycosylated VNTR peptides of different length (one to five repeats). No binding increment with increasing number of repeats is observed. Their binding is independent of DTR glycosylation (not shown here, see Tables II and VI). The epitope of mAb MF11 is not sufficiently presented in the 20-mer.

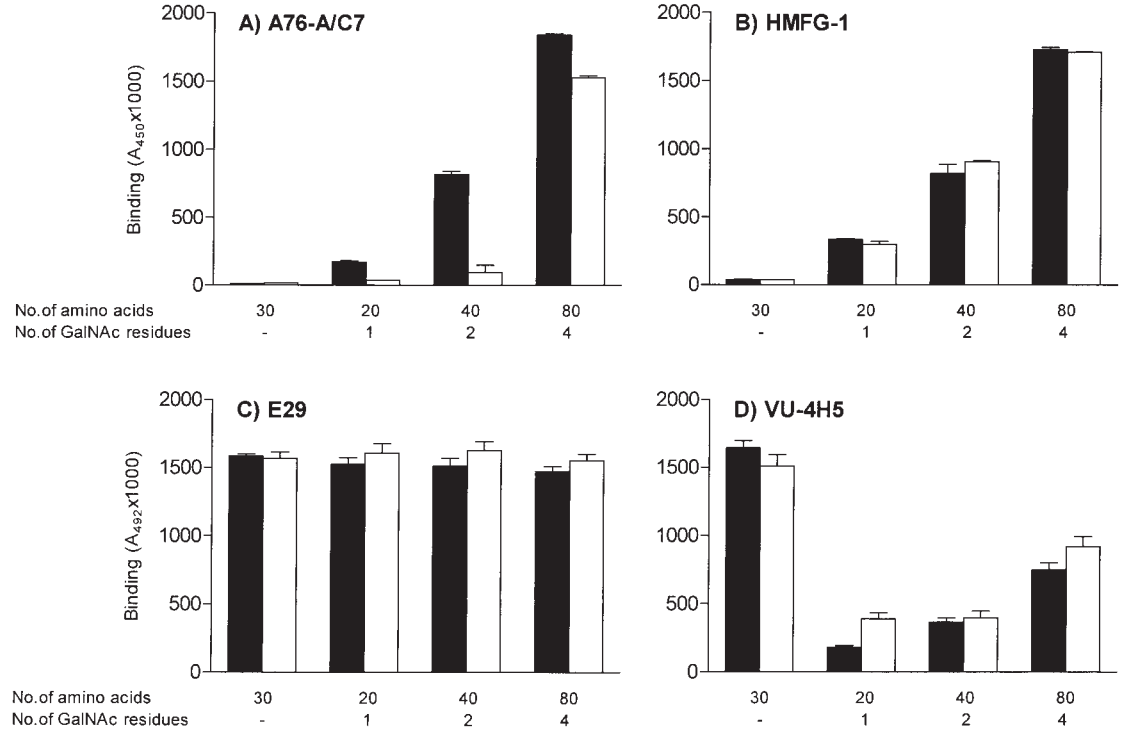

Fig. 4. Influence of carbohydrate-specific periodate oxidation on binding patterns of MUC1 antibodies to unglycosylated (first column) and glycosylated VNTR peptides of different length (one, two, or four repeats). Characteristics of antibodies: **A**: glycosylation-dependent; **B** and **C**: glycosylation-independent; **D**: glycosylation-inhibited. Black columns: control incubation; white columns: periodate treatment. Only in case of a glycosylation-dependent antibody (**A**) an influence of periodate oxidation on binding is observed, which is partially compensated for by a peptide length of at least four repeats.

conditions (1 h at 0°C), leaves GalNAc *O*-glycosidically bound to the peptide backbone intact (Mort and Lamport, 1977), resulting in structures similar to the tumor epitope defined by us (see *Discussion*).

Among the other parameters, some interesting correlations to the two subgroups (glycosylation-dependent [A, Table IV] versus glycosylation-independent mabs [B, Table V]) were also found. First, the length dependency is more pronounced in subgroup A compared to B. Second, in subgroup A the mean peptide length of the epitopes (8.4 amino acids) was longer than in subgroup B (5.6 amino acids). Both differences may be interpreted as indicators of the prevalence of conformation epitopes rather than sequence epitopes in glycosylation-effected MUC1 antibodies. Efficiency points in recognizing shed tumor MUC1 according to Norum *et al.* (1998) were also differing

between groups A and B. The numerical sum of all points of group A was 435 as compared to 218 in group B, although individual scores varied considerably. This can be explained by the fact that shed tumor MUC1 is obviously different from cell-bound tumor MUC1 because it is only weakly detected by some mAbs (unpublished data). No correlation was found to isotypes.

Discussion

In continuing earlier studies on the influence of glycosylation on the binding of antibodies to peptide epitopes of MUC1 (Cao *et al*., 1997a,b, 1998; Cao and Karsten, 2001; Karsten *et al*., 1998), we reexamined, with a large panel of 22 mAbs to the immunodominant DTR motif of MUC1, their binding patterns with respect to peptide length (i.e., the number of tandem repeats) and DTR glycosylation. Essential results were that the impacts of length and glycosylation on antibody binding, which at first hand appeared to be similar, were in fact different (and in a subgroup of mAbs additive), and that the presence or absence of the glycosylation effect split the antibody population into two groups. The most astonishing result was that antibodies generated from tumor-derived MUC1 were all glycosylation-influenced, whereas those generated from nontumor MUC1 were independent of DTR glycosylation in their binding behavior. We interpret this as clear evidence that tumor MUC1 contains a tumor-specific conformation epitope of the structure: ...PDT*RP..., where T* is *O*-glycosylated with GalNAcα1- or a similar short, nonsialylated glycan, such as Galβ1-3GalNAcα1 (core 1).

Our conclusion is supported by a recent study in which the fine specificities of anti-MUC1 antibodies in human sera were reported. Anti-MUC1 antibodies from breast cancer patients revealed a preference for glycosylated peptides (Von Mensdorff-Pouilly *et al*., 2000a).

The present study was done with GalNAcα1-substituted peptides; from our earlier data (Karsten *et al*., 1998) it can be inferred that Galβ1-3GalNAcα1- instead of GalNAcα1- is equally effective with respect to an enhanced binding of antibodies of the respective group. Both are typical glycans of tumor MUC1 (Goletz *et al*., 2003; Lloyd *et al*., 1996). However the glycan moiety itself is not directly recognized by the MUC1 antibodies. This can be inferred from the following: (1) all mAbs included in this study recognize defined peptide sequences (Price *et al*., 1998) and bind significantly to unglycosylated oligomeric MUC1 tandem repeats; (2) binding increments by glycosylation are similar with either GalNAcα1- or Galβ1-3GalNAcα1-, which are two immunologically completely different entities; (3) the mAbs do not bind to either of the glycans in enzyme-linked immunosorbent assay (ELISA) (data not shown); and (4) structural studies with mAb SM3 (glycosylation-dependent, Table II) revealed no evidence for carbohydrate involvement in binding (Möller *et al*., 2002).

A number of nuclear magnetic resonance (NMR) studies have dealt with the secondary structure of the DTR motif (Fontenot *et al*., 1993, 1995; Grinstead *et al*., 2002; Kirnarsky *et al*., 2000; Scanlon *et al*., 1992; Schuman *et al*., 2003; Tendler, 1990). In one case, the crystal structure of an antibody-peptide complex has been accomplished (Dokurno *et al*., 1998). The influence of peptide elongation (from one to three nonglycosylated tandem repeats) was described as leading to a knoblike, type I β-turn conformation of the region around DTR (Fontenot *et al*., 1993, 1995). The effect of DTR glycosylation on its conformation has recently been investigated (Kirnarsky *et al*., 2000; Möller *et al*., 2002), albeit only with short glycopeptides. Attachment of GalNAcα1-*O*- shifts the conformation of the DTR motif from the type I β-turn toward a more rigid and extended state (Schuman *et al*., 2003). This supports our suggestion that length and glycosylation effects lead to different conformations of this region.

O-glycosylation with GalNAc has also been shown to result in a more rigid conformation in case of peptides other than MUC1 (Huang *et al*., 1997; Live *et al*., 1999).

Whereas a β-turn within the DTR motif may explain the immunodominance of this region in case of the unglycosylated peptide (Fontenot *et al*., 1993, 1995; Schuman *et al*., 2003), our data suggest that this is *not* the tumor-specific MUC1 epitope. The correlations found by us imply that tumor MUC1 contains the glycosylated DTR, which is in agreement with biochemical data (Müller *et al*., 1999), and indicate the prevalence of conformation epitopes rather than sequence epitopes among DTR-specific antibodies generated against tumor MUC1. We hypothesize that the tumor-specific MUC1 epitope is a certain conformation of the ...PDTRP... epitope. Its exact structure, of course, cannot be deduced from our data. It should have similarity to that described by Schuman *et al*. (2003), but may be modified by the length effect. In any case, the tumor-specific MUC1 epitope is clearly different from what it hitherto was believed to be. The oligomeric MUC1 glycopeptides described here could provide an excellent subject for structural studies, which may be able to elucidate the complex interplay of glycosylation and oligomerization on the conformation of this immunologically important site of tumor MUC1.

Our results should be relevant to MUC1-based immunotherapies. Antibodies that specifically recognize the epitope described here are less prone to bind to normal MUC1. This explains, at least in part, the already described more or less tumor-specific histological staining pattern of some MUC1 antibodies, for example, SM3 (Burchell *et al*., 1987) or A76-A/C7 (Cao *et al*., 1997a). However, it is evident from this and earlier studies (Cao and Karsten, 2001; Norum *et al*., 1998) that even nominally similar antibodies reveal astonishing differences in their fine specificity. In addition, the selection of an individual antibody for adjuvant immunotherapy depends on a number of other parameters, too. The case for cancer vaccines is more straightforward. On the one hand, the DTR motif is well known as a B cell epitope. The presence of anti-MUC1 antibodies in the serum has been found beneficial for breast cancer patients (Von Mensdorff-Pouilly *et al*., 2000b). On the other hand, among known MUC1 T cell epitopes (Brossart *et al*., 1999; Feuerer *et al*., 2001), the DTR region has also been reported (Gad *et al*., 2003; Mukherjee *et al*., 2000). Most recently, Apostolopoulos *et al*. (2003) have actually shown that glycosylation with GalNAc at the DTR motif leads to a high-affinity binding to a murine major histocompatibility

class I molecule, H-2Kb, and that the glycosylated MUC1 peptide is able to evoke strong specific T cell responses *in vitro* and *in vivo*. Similar results have been obtained with other proteins (e.g., a lysozyme peptide; Harding *et al*., 1993). In conclusion, we propose that MUC1-based cancer vaccines should contain the ...PDTRP... sequence *O*-glycosylated at the threonine with Tn or TF (i.e., in a conformation corresponding to tumour MUC1).

Materials and methods

Antibodies

Twenty-two mAbs toward the immunodominant DTR motif of MUC1 were selected from the TD-4 Workshop panel (Rye and Price, 1998) for this study. In addition, two MUC1 mAbs with epitopes distant from this motif were included. The antibodies selected, their isotypes, and the immunogens employed for their generation (taken from Rye and Price, 1998) are listed in Table I together with their nominal epitope sequences as determined by the TD-4 Workshop (Rye and Price, 1998).

Peptides and glycopeptides

Three sets of synthetic peptides/glycopeptides, all based on the MUC1 tandem repeat sequence, were employed for three different series of experiments.

Series I. Unglycosylated 20-mers and their oligomers of the type $[VTSAPDTRPAPGSTAPPAHG]_n$, with $n = 1$, 3, 4, 5, and 6, were obtained from Dr. J. Hilgers (Department of Obstetrics and Gynaecology, Free University, Amsterdam, The Netherlands). In the peptides with $n = 3$–6, R was missing in the second repeat.

Series II. A glycosylated 30-mer with the sequence APPAHGVTSAPDT[GalNAcα]RPAPGSTAPPAHGVTSA and its unglycosylated counterpart were synthesized by Biosyntan GmbH (Berlin-Buch). In addition, the same pair of glycosylated versus unglycosylated peptide was prepared in an N-terminally biotinylated form, which then could be coated more quantitatively on streptavidin microtiter plates (see later description). Biotinylation (with 6-aminohexanoic acid as N-terminal spacer) was performed as follows. Biotin was preactivated with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate and N-methylmorpholine in N-methylpyrrolidone for 10 min, and this mixture together with the solid phase containing the peptide/glycopeptide was stirred for 2 h.

Series III. A novel series of glycosylated MUC1 tandem repeat peptides of different length of the sequence $A[HGVTSAPDT(GalNAc\alpha)RPAPGSTAPPA]_n$, with $n = 1$–5 (TR1a–TR5a), was synthesized (Table III).

The synthesis of the glycopeptides was conducted as a glycopeptide solid phase synthesis on Wang resin via the 9-fluorenyl-methoxycarbonyl (FMOC)[3] technique. The amino acids were coupled as FMOC-fluorophenylesters, and the FMOC-ThrGalNAc building blocks with the help of *O*-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium tetrafluoroborate. The synthesis was started with a larger amount of resin (2.5 g) until TR1a was reached. One-fifth of the resin was then removed, and the synthesis was continued with the remaining resin via TR2a, TR3a, TR4a, and TR5a. After each of the intermediates had been achieved, a portion of the resin was removed, and the majority of the resin was used for the next step. The deacetylation of the saccharide was conducted with methanol/hydrazine 5:1 except for TR4a and TR5a, where 2-propanol/hydrazine 5:1 was used. Cleavage from the resin was achieved with 0.1% aqueous trifluoroacetic acid (95%). The purification of the substances was carried out by high-pressure liquid chromatography on reversed phase columns (RP18) in a gradient mode with water/acetonitrile as mobile phase. Structural elucidation was performed with NMR spectroscopy, and the molecular weights were determined by matrix-assisted laser desorption and ionization mass spectrometry.

Enzyme immunoassays

ELISAs were performed as follows.

Experimental series I (length effect). Polystyrene microtiter plates of tissue culture type (Nunc, Wiesbaden, Germany) were coated with 50 µl per well of a solution of 10 µg/ml of peptide (from series I) in 0.1 M carbonate buffer, pH 9.6, overnight at 37°C to dryness. Antigens to be compared were always coated on the same plate. After three washes with phosphate buffered saline (PBS)/0.05% Tween 20, the purified antibodies were added (50 µl, 10 µg/ml) in PBS/Tween containing 1% bovine serum albumin (BSA), and incubated for 2 h in a moist chamber at 37°C. After three washings as before, the plates were incubated with peroxidase-labeled rabbit anti-mouse immunoglobulin serum (P260, Dako, Hamburg, Germany) diluted 1:2000 in PBS/BSA for 1.5 h at 37°C. After three final washings, color development was accomplished with o-phenylenediamine, and the absorbance (A) measured with a Spectra plate reader (SLT Labinstruments, Salzburg, Austria) at 492 nm. Blank values were subtracted, and means were calculated. From the means, ratio values $A_{100\text{-mer}}$:$A_{20\text{-mer}}$ were calculated.

Experimental series II (glycosylation effect). Experimental details were similar to series I except that the 30-mer peptide and glycopeptide from series II were employed, and water was used for coating instead of carbonate buffer to avoid β-elimination during the drying process. To exclude the possibility that glycopeptides and peptides sticked differently to the plastic, a parallel series of experiments with biotinylated (glyco)peptides was performed. In this case, the protocol was modified as follows. Streptavidin-precoated microtiter plates (BioTeZ, Berlin-Buch) were coated with 100 µl per well containing 0.5 µg/ml of the biotinylated antigen in PBS/BSA (1 h at room temperature). The following steps were as described except that 100 µl volumes were used throughout and the concentrations of the primary and second antibodies were halved. Blank values were subtracted, and means were calculated. From the means, ratio values $A_{\text{glycosylated peptide}}$:$A_{\text{unglycosylated peptide}}$ were calculated.

Experimental series III (combined effects). Experimental details were similar to series I except that glycopeptides of series III were employed and water was used for coating instead of carbonate buffer. The concentration of the primary antibodies was varied between 0.1 and 5 μg/ml. Evaluation of binding patterns was generally based on data obtained at 5 μg/ml, although in most cases the overall patterns were found consistent for a given mAb within the whole concentration range. In some experiments the antigen concentration was also varied. Best results were obtained at 10 μg/ml. In some experiments, 3,3′,5,5′-tetramethylbenzidine instead of o-phenylenediamine was used as substrate for peroxidase, and A was measured at 450 nm.

In all ELISA experiments, coating was done on a weight per ml basis to approximate equimolar concentrations of tandem repeats.

In some cases, carbohydrate-selective periodate oxidation of the coated antigens (10 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 4.5, for 1 h at 25°C, followed by reduction of aldehydes by 50 mM $NaBH_4$) was performed as described (Woodward *et al*., 1985).

Values were generated in duplicates and repeated at least three times. Graphs were prepared with the GraphPad Prism 3.0 program (GraphPad Software, San Diego, CA). Error bars indicate SE.

Acknowledgments

We thank Dr. Jo Hilgers (Amsterdam) for providing non-glycosylated MUC1 peptides, Anneliese Koberling for excellent technical assistance, and Dr. Martin Lipp (Max Delbrück Centre for Molecular Medicine) for critically reading the manuscript. The study was performed as an extension of the ISOBM TD-4 International Workshop on Monoclonal Antibodies against MUC1.

Abbreviations

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FMOC, 9-fluorenyl-methoxycarbonyl; mAb, monoclonal antibody; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline.

References

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

Brockhausen, I. (1999) Pathways of *O*-glycan biosynthesis in cancer cells. *Biochim. Biophys. Acta*, **1473**, 67–95.

Brossart, P., Heinrich, K.S., Stuhler, G., Behnke, L., Reichardt, V.L., Stevanovic, S., Muhm, A., Rammensee, H.-G., Kanz, L., and Brugger, W. (1999) Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. *Blood*, **93**, 4309–4317.

Burchell, J., Gendler, S., Taylor-Papadimitriou, J., Girling, A., Lewis, A., Millis, R., and 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. M., Mungul, A., and Taylor-Papadimitriou, J. (2001) O-linked glycosylation in the mammary gland: changes that occur during malignancy. *J. Mamm. Gland Biol. Neopl.,* **6**, 355–363.

Cao, Y. and Karsten, U. (2001) Binding patterns of 51 monoclonal antibodies to peptide and carbohydrate epitopes of the epithelial mucin (MUC1) on tissue sections of adenolymphomas of the parotid (Warthin's tumours): role of epitope masking by glycans. *Histochem. Cell Biol.*, **115**, 349–356.

Cao, Y., Blohm, D., Ghadimi, B. M., Stosiek, P., Xing, P.-X., and Karsten, U. (1997a) Mucins (MUC1 and MUC3) of gastrointestinal and breast epithelia reveal different and heterogeneous tumor-associated aberrations in glycosylation. *J. Histochem. Cytochem.*, **45**, 1547–1557.

Cao, Y., Schlag, P. M., and Karsten, U. (1997b) Immunodetection of epithelial mucin (MUC1, MUC3) and mucin-associated glycotopes (TF, Tn, and sialosyl-Tn) in benign and malignant lesions of colonic epithelium: apolar localization corresponds to malignant transformation. *Virchows Archiv.*, **431**, 159–166.

Cao, Y., Karsten, U., and Hilgers, J. (1998) Immunohistochemical characterization of a panel of 56 antibodies with normal human small intestine, colon, and breast tissues. *Tumor Biol.*, **19**(suppl 1), 88–99.

Dokurno, P., Bates, P.A., Band, H.A., Stewart, L.M.D., Lally, J.M., Burchell, J.M., Taylor-Papadimitriou, J., Snary, D., Sternberg, M.J.E., and Freemont, P.S. (1998) Crystal structure at 1.95 Å 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, E.-F., Bastert, G., Diel, I. J., Pedain, C., Oberniedermayr, M., Schirrmacher, V., and Umansky, V. (2001) Therapy of human tumors in NOD/SCID mice with patient-derived reactivated memory T cells from bone marrow. *Nature Med.*, **7**, 452–458.

Finn, O.J., Jerome, K.R., Henderson, R.A., Pecher, G., Domenech, N., Magarian-Blander, J., and Barratt-Boyes, S.M. (1995) MUC-1 epithelial tumor mucin-based immunity and cancer vaccines. *Immunol. Rev.*, **145**, 61–89.

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

Fontenot, J.D., Mariappan, S.V.S., Catasti, P., Domenech, N., Finn, O.J., and Gupta, G. (1995) Structure of a tumor associated antigen containing a tandemly repeated immunodominant epitope. *J. Biomol. Struct. Dyn.*, **13**, 245–260.

Gad, M., Jensen, T., Gagne, R., Komba, S., Daugaard, S., Kroman, N., Meldal, M., and 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.

Goletz, S., Cao, Y., Danielczyk, A., Ravn, P., Schöber, U., and Karsten, U. (2003) Thomsen-Friedenreich antigen: the ìhiddenî tumor antigen. *Adv. Exp. Med. Biol.*, **535**, 147–162.

Grinstead, J.S., Koganty, R.R., Krantz, M.J., Longenecker, B.M., and Campbell, A.P. (2002) Effect of glycosylation on MUC1 humoral immune recognition: NMR studies of MUC1 glycopeptide-antibody interactions. *Biochemistry*, **41**, 9946–9961.

Harding, C.V., Kihlberg, J., Elofsson, M., Magnusson, G., and Unanue, E.R. (1993) Glycopeptides bind MHC molecules and elicit specific T cell responses. *J. Immunol.*, **151**, 2419–2425.

Hayes, D.F., Zurawski, V.R., and Kufe, D.W. (1986) Comparison of circulating CA15–3 and carcinoembryonic antigen in patients with breast cancer. *J. Clin. Oncol.*, **4**, 1542–1550.

Huang, X., Barchi, J.J., Lung, F.-D.T., Roller, P.P., Nara, P.L., Muschik, J., and Garrity, R.R. (1997) Glycosylation affects both the three-dimensional structure and antibody binding properties of the HIV-1_{IIIB} GP120 peptide RP135. *Biochemistry*, **36**, 10846–10856.

Karsten, U., Diotel, C., Klich, G., Paulsen, H., Goletz, S., Müller, S., and Hanisch, F.-G. (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.

Kirnarsky, L., Prakash, O., Vogen, S.M., Nomoto, M., Hollingsworth, M.A., and Sherman, S. (2000) Structural effects of O-glycosylation on a

15-residue peptide from the mucin (MUC1) core protein. *Biochemistry*, **39**, 12076–12082.

Live, D.H., Williams, L.J., Kuduk, S.D., Schwarz, J.B., Glunz, P.W., Chen, X.-T., Sames, D., Kumar, R.A., and Danishewsky, S.J. (1999) Probing cell-surface architecture through synthesis: an NMR-determined structural motif for tumor-associated mucins. *Proc. Natl Acad. Sci. USA*, **96**, 3489–3493.

Lloyd, K.O., Burchell, J., Kudryashov, V., Yin, B.W.T., and 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. *J. Biol. Chem.*, **271**, 33325–33334.

Möller, H., Serttas, N., Paulsen, H., Burchell, J.M., Taylor-Papadimitriou, J., and Meyer, B. (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.

Mort, A.J. and Lamport, D.T.A. (1977) Anhydrous hydrogen fluoride deglycosylates glycoproteins. *Anal. Biochem.*, **82**, 289–309.

Mukherjee, P., Ginardi, A.R., Madsen, C.S., Sterner, C.J., Adriance, M.C., Tevethia, M.J., and Gendler, S.J. (2000) Mice with spontaneous pancreatic cancer naurally develop MUC-1-specific CTLs that eradicate tumors when adoptively transferred. *J. Immunol.*, **165**, 3451–3460.

Müller, S., Goletz, S., Packer, N., Gooley, A., Lawson, A.M., and Hanisch, F.-G. (1997) Localization of *O*-glycosylation sites on glycopeptide fragments from lactation-associated MUC1. All putative sites within the tandem repeat are glycosylation targets *in vivo*. *J. Biol. Chem.*, **272**, 24780–24793.

Müller, S., Alving, K., Peter-Katalinic, J., Zachara, N., Gooley, A.A., and Hanisch, F.-G. (1999) High density *O*-glycosylation on tandem repeat peptide from secretory MUC1 of T47D breast cancer cells. *J. Biol. Chem.*, **274**, 18165–18172.

Norum, L.F., Varaas, T., Kierulf, B., and Nustad, K. (1998) Carcinoma-associated MUC1 detected by immunoradiometric assays. *Tumor Biol.*, **19**(suppl 1), 134–146.

Price, M.R., Rye, P.D., Petrakou, E., Murray, A., Brady, K., Imai, S., Haga, S., Kiyozuka, Y., Schol, D., Meulcnbroek, M.F.A., and others. (1998) Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. *Tumor Biol.,* **19**(suppl. 1), 1–20.

Rye, P.D. and Price, M.R. (1998) ISOBM TD-4 International workshop on monoclonal antibodies against MUC1. *Tumor Biol.*, **19**(suppl 1), 1–152.

Safi, F., Kohler, I., Röttinger, E., and Beger, H.-G. (1991) The value of the tumor marker CA 15–3 in diagnosing and monitoring breast cancer. *Cancer*, **68**, 574–582.

Scanlon, M.J., Morley, S.D., Jackson, D.E., Price, M.R., and Tendler, S.J.B. (1992) Structural and computational investigations of the conformation of antigenic peptide fragments of human polymorphic epithelial mucin. *Biochem. J.*, **284**, 137–144.

Schuman, J., Koganty, R.R., Longenecker, B.M., and Campbell, A.P. (2003) Probing the conformational and dynamical effects of *O*-glycosylation within the immunodominant region of a MUC1 peptide tumor antigen. *J. Pept. Res.*, **61**, 91–108.

Taylor-Papadimitriou, J., Burchell, J., Miles, D.W., and Dalziel, M. (1999) MUC1 and cancer. *Biochim. Biophys. Acta*, **1455**, 301–313.

Tendler, S.J.B. (1990) Elements of secondary structure in a human epithelial mucin core peptide fragment. *Biochem. J.*, **267**, 733–737.

Von Mensdorff-Pouilly, S., Petrakou, E., Kenemans, P., Van Uffelen, K., Verstraeten, A.A., Snijdewint, F.G.M., Van Kamp, G.J., Schol, D.J., Reis, C.A., Price, M.R., and others. (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, A.A., Kenemans, P., Snijdewint, F.G.M., Kok, A., Van Kamp, G.J., Paul, M.A., Van Diest, P.J., Meijer, S., and Hilgers, J. (2000b) Survival in early breast cancer patients is favorably influenced by a natural humoral immune response to polymorphic epithelial mucin (MUC1). *J. Clin. Oncol.*, **18**, 574–583.

Woodward, M.P., Young, W.W., and Bloodgood, R.A. (1985) Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *J. Immunol. Methods*, **78**, 143–153.