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Characterization of an immunodominant cancer-specific O-glycopeptide epitope in murine podoplanin (OTS8)

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Abstract Auto-antibodies induced by cancer represent promising sensitive biomarkers and probes to identify immunotherapeutic targets without immunological tolerance. Surprisingly few epitopes for such auto-antibodies have been identified to date. Recently, a cancer-specific syngeneic murine monoclonal antibody 237, developed to a spontaneous murine fibrosarcoma, was shown to be directed to murine podoplanin (OTS8) with truncated Tn O-glycans. Our understanding of such cancer-specific auto-antibodies to truncated glycoforms of glycoproteins is limited. Here we have investigated immunogenicity of a chemoenzymatically produced Tn-glycopeptide derived from the putative murine podoplanin O-glycopeptide epitope. We found that the Tn O-glycopeptide was highly immunogenic in mice and produced a Tn-glycoform specific response with no reactivity against unglycosylated peptides or the O-glycopeptide with extended O-glycan (STn and T glycoforms). The immunodominant epitope was strictly dependent on the peptide sequence, required Tn at a specific single Thr residue (Thr^{77}), and antibodies to the epitope were not found in naive mice. We further tested a Tn O-glycopeptide library derived from human podoplanin by microarray analysis and demonstrated that the epitope was not conserved in man. We also tested human cancer sera for potential auto-antibodies to similar epitopes, but did not detect such antibodies to the Tn-library of podoplanin. The reagents and methods developed will be valuable for further studies of the nature and timing of induction of auto-antibodies to distinct O-glycopeptide epitopes induced by cancer. The results demonstrate that truncated O-glycopeptides constitute highly distinct antibody epitopes with great potential as targets for biomarkers and immunotherapeutics.

Keywords Autoantibody · O-glycosylation · O-glycopeptide · Podoplanin

Abbreviations

KLH	keyhole limpet hemocyanin
M/PAb	monoclonal/polyclonal antibody
ELISA	enzyme linked immunosorbent assay
HPA/HAA	Helix pomatia/aspersa lectin

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Introduction

Cancer-induced auto-antibodies represent an interesting probe to identify cancer antigens for which immunological tolerance is non-existent or broken. The presence of auto-antibodies to cancer was originally demonstrated using autologous cancer tissue or cells [1, 2], and identification of the molecular

nature of the recognized cancer antigens have followed several different strategies. A common feature of the strategies employed in the past has been difficulty with identification of protein antigen epitopes affected by aberrant glycosylation and other posttranslational modifications. In fact most high through-put screening strategies for auto-antibodies including expressed cDNA libraries (SEREX) [3], protein and peptide arrays [4, 5], random or designed phage displays [6], and more recently self-assembling protein arrays [7, 8] completely ignore posttranslational modifications, and in particular aberrant glycosylation. Since most cell membrane and secreted proteins are glycoproteins, it is likely that antibody epitopes that incorporate, or are otherwise affected by, glycosylation may be largely missed.

We and others have developed and characterized a number of monoclonal antibodies (MAb) that specifically recognize distinct O-glycopeptides [9–14], and found that these antibodies have distinct specificity for the combined peptide sequence and the O-glycoform. Of particular interest are antibodies that react with cancer-associated truncated glycoforms of proteins. We recently identified an immunodominant epitope in the tandem repeat region of the human mucin MUC1, which is comprised of the peptide sequence -GSTAP- with one or two Tn O-glycans (GalNAcα1-O-Ser/Thr) in the 20mer tandem repeat sequence (HGVTSAPDTRPAPGSTAPPA, acceptor sites underlined) [10, 15]. This epitope is virtually cancer specific and not covered by immunological tolerance in man [10, 15, 16], and in preliminary studies we have identified auto-antibodies to this epitope in cancer patients and not in healthy individuals [17]. Our understanding of the fine specificity of such glycopeptide antibodies, the molecular nature of the recognized epitopes, and regulation of immunity to these epitopes is limited, and availability of animal model systems would be highly valuable.

Recently, a syngeneic cancer-specific MAb 237 against an aggressive spontaneous fibrosarcoma Ag104A was shown to react with the Tn glycoform of murine podoplanin (OTS8)

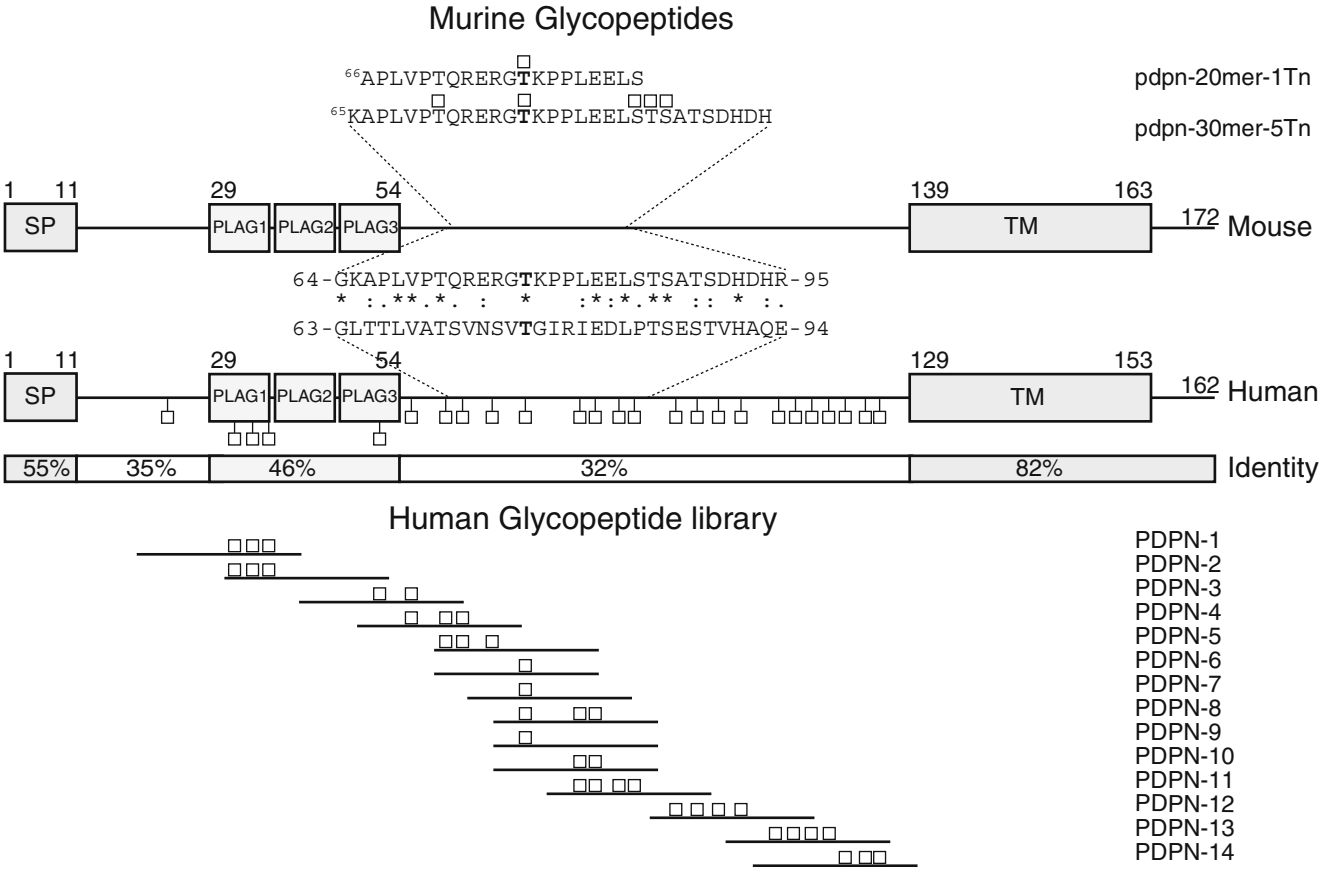


Fig. 1 Schematic presentation of the murine and human podoplanin. The overall sequence identity between the two species is low; however, the transmembrane domain (TM), the signal peptide (SP), and the three platelet aggregation-stimulation domains (PLAG1-3) are well conserved, as determined by a ClustalW alignment. The sequence in the area of murine Thr^{77} is very different in the two species as depicted in the figure. □— indicates O-glycosylation sites predicted by the NetOGlyc prediction tool [22] used to design the human glycopeptide library (PDPN1-14) applied in this study. Thr^{70} is not predicted to be O-glycosylated by the current version of NetOGlyc, but this site was still included in the library on PDPN-5. The sequences of the two murine glycopeptides (pdpn-20/30mer) are shown in the figure as well

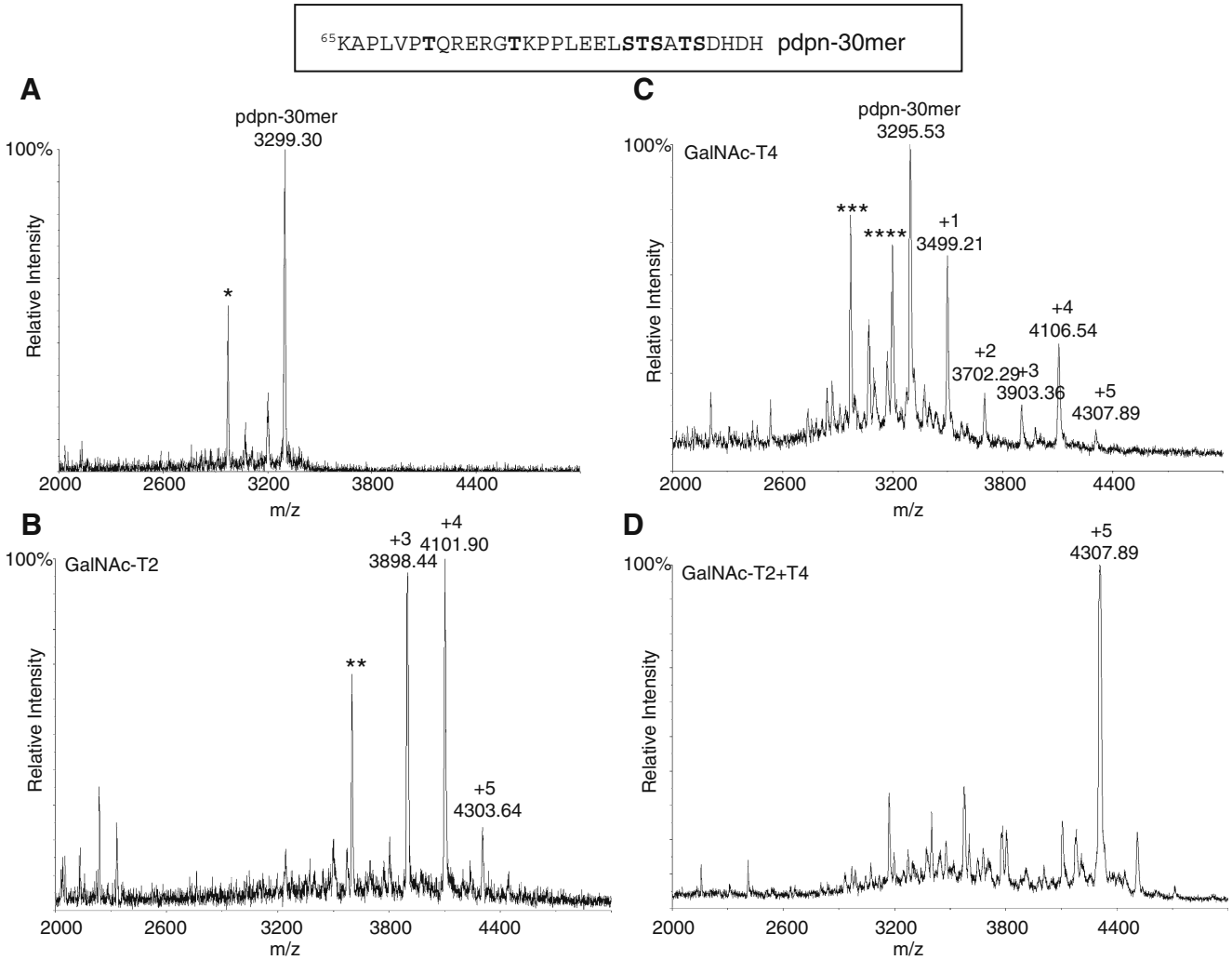


Fig. 2 MALDI-TOF-MS analysis of chemoenzymatic synthesized pdpn-30mer-Tn. pdpn-30mer (**a**) containing 7 possible glycosylation sites (bold) was glycosylated with (**b**) recombinant polypeptide GalNAc-T2, (**c**) GalNAc-T4 or (**d**) GalNAc-T2 and -T4. * represents peaks not assigned to the actual pdpn-30mer (m/z *2969.89, **3600.57, ***2970.88, ****3199.31)

[18]. The antibody reacted with podoplanin only when expressed in Ag104A cells, and the molecular basis for this finding was associated with truncated O-glycosylation. Thus, it was found that the chaperone cosmc was mutated in Ag104A cells, resulting in inactive T-synthase (core 1 β3-galactosyltransferase, which adds galactose to the Tn antigen to generate the T-antigen; Galβ1-3GalNAcα1-O-Ser/Thr) and hence general truncation of O-glycosylation mainly to short Tn structures [19, 20]. The binding epitope of MAb 237 was further narrowed down to a protein sequence with at least one O-glycan at residue Thr^{77} [18] (Structure and sequence depicted in Fig. 1). Loss of cosmc is expected to widely affect O-glycosylation on many glycoproteins, which suggests that the identified podoplanin auto-antibody epitope is immunodominant, although other similar epitopes may have been deselected by the experimental design of the study.

In order to provide more insight into the nature of such aberrant immunodominant glycopeptide epitopes, we have further studied the murine podoplanin epitope and its immunogenicity in mice. We chemoenzymatically produced a 30mer Tn-glycopeptide covering the Thr^{77} residue and used this, coupled to KLH, to immunize mice and characterize specificity of the polyclonal antibody (PAb) response. We demonstrated that the epitope was highly immunogenic and strictly confined to a Tn-glycopeptide sequence with no cross reaction with the unglycosylated peptide, the glycopeptide with different O-glycans, or the Tn hapten O-glycan.

Materials and methods

Synthesis and analysis of glycopeptides

A 30mer peptide designed from murine podoplanin covering residues 65–94 (pdpn-30mer) was custom

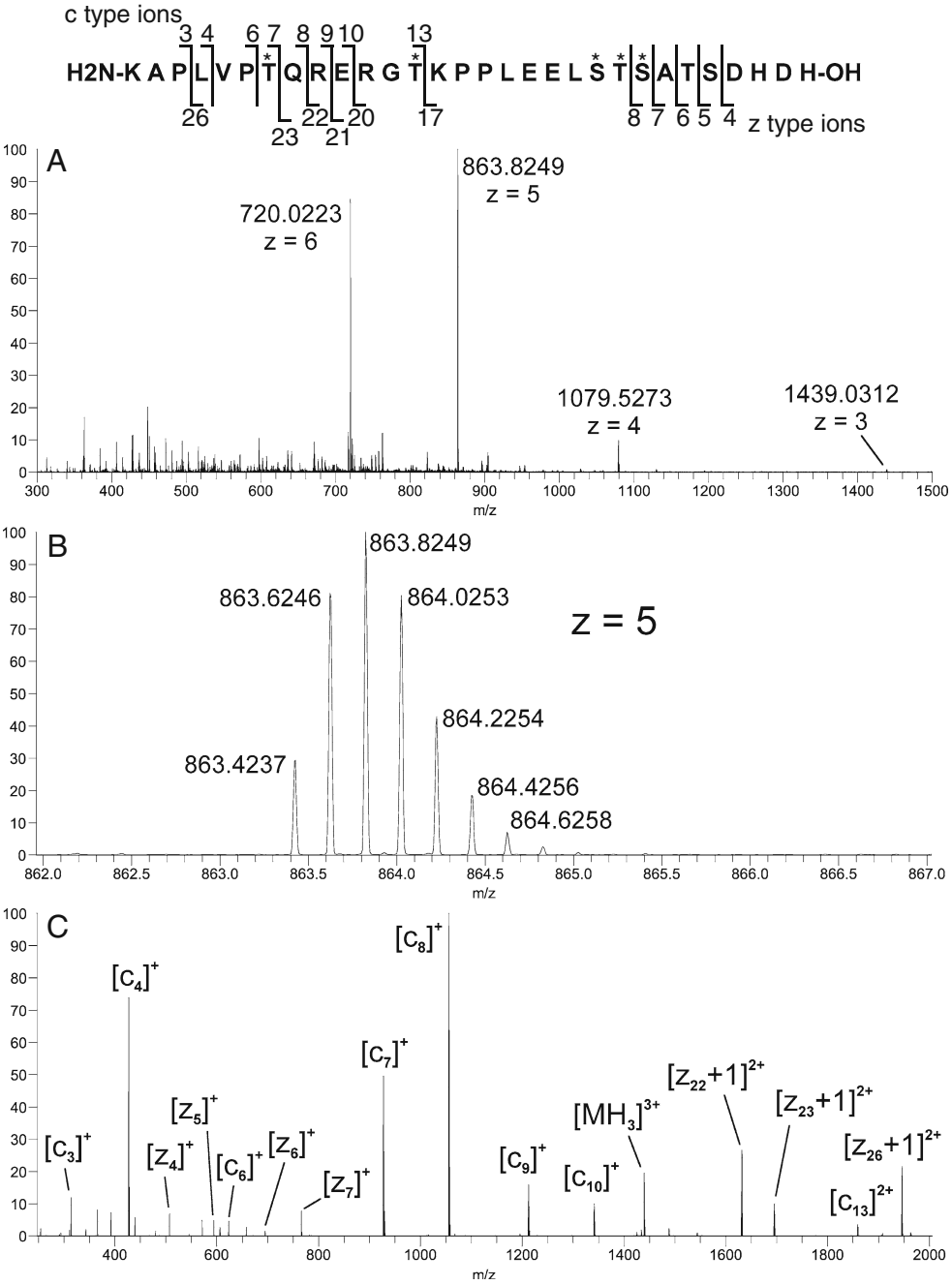


Fig. 3 Characterization of pdpn-30mer-5Tn by ETD in the LTQ-Orbitrap. (**a**) MS^1 of pdpn-30mer-5Tn; (**b**) expansion of MH_5^{5+} showing exact *m/z* and distribution of resolved isotope peaks; (**c**) ETD-MS^2 of precursor MH_5^{5+} ion. The fragmentation pattern is consistent with glycosylation of $Thr^{7,13,22}$ and $Ser^{21,23}$ with a HexNAc residue (*). Detection of $z\cdot_5^+$, $z\cdot_6^+$, and $z\cdot_7^+$ exclusively without HexNAc indicates the absence of glycosylation at sites Thr^{25} and Ser^{26}. All detected fragment ions are listed in Table 1

synthesized by Schafer-N (Denmark), and glycosylated *in vitro* using purified recombinant human UDP-GalNAc: polypeptide *N*-Acetylgalactosaminyltransferases GalNAc-T2 and/or -T4 as previously described [10]. The reaction was monitored by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and the final product characterized by electrospray ionization-linear ion trap-Fourier transform mass spectrometry (ESI-LIT-FT-MS) in an LTQ-Orbitrap XL hybrid spectrometer (Thermo-Scientific, Bremen, Germany), equipped with electron transfer dissociation (ETD) [21] for peptide sequence analysis by MS/MS (MS^2) with retention of glycan site-specific fragments. The sample was dissolved in methanol-water (1:1) containing 1% formic acid, and introduced by direct infusion via a TriVersa NanoMate ESI-Chip interface (Advion BioSystems, Ithaca, NY, USA), at a flow rate ~100 nL/min and 1.4 kV spray voltage. Mass spectra were acquired in positive ion FT mode at a nominal resolving power of 30,000; in addition to the normal calibration process, a polydimethylcyclosiloxane ion (*m/z* 445.1200) was used as an internal reference in MS^1. Several ETD-MS^2 spectra

were acquired on the MH_5^{5+} molecular precursor ion cluster using an isolation width of 3 mu; normalized collision energy, 35%; activation Q, 0.25; activation times of either 100 or 200 ms; and supplemental activation, 20%. Experimental MS^1 spectra were analyzed by comparison to exact *m/z* values calculated for the observed molecular ion charge states, using the known podoplanin peptide sequence incremented with 5 HexNAc residues; ETD-MS^2 spectra were analyzed by comparison with theoretical c- and z·- fragment *m/z* values calculated for all positional combinations of 5 HexNAc residues distributed on the 7 potential S and T glycosylation sites in the sequence. Calculations were performed using www-based Protein Prospector (http://prospector.ucsf.edu/) MS-Isotope and MS-Product software routines.

Two shorter 20mer (glyco) peptides covering residues 66–85 were synthesized using standard Fmoc solid phase peptide synthesis (SPPS) with and without a GalNAc residue at Thr^{12} (pdpn-20mer/pdpn-20mer-1Tn). The pdpn-20mer-1Tn glycopeptide containing an aminohexanoic acid (Ahx) linker was purchased from Sigma. The STn and T glycoforms of pdpn-20mer-1Tn were produced by *in vitro* glycosylation with recombinant human ST6GalNAc-I or a recombinant T-synthase from *D. melanogaster*, dC1Gal-T1, as previously described [15]. The products were characterized by MALDI-TOF as well as ELISA using anti-STn and anti-T monoclonal antibodies and the anti-Tn lectin from *Helix pomatia* (HPA) confirming glycosylation to be essentially completely converted to STn and T glycoforms. When required, glycopeptides were purified by high performance liquid chromatography (HPLC) on an 1100 Hewlet Packard system (Avondale, PA) using a Hypersil Gold 1.9 μm column (50×2.5 mm) (Thermo Scientific) or a Zorbax 300SB-C18 column (9.4 mm×25 cm) (Agilent Technologies, Alo Alto, CA) with 0.1% TFA and a gradient of 0–100% acetonitrile.

Fourteen GalNAc glycopeptides (PDPN-1-14) covering putative O-glycosylation sites in human podoplanin predicted by the NetOGlyc algorithm [22] were designed and synthesized by multi-well SPPS carried out using stepwise blocking by acetylation to allow direct printing without purification [O. Blixt *et al*. manuscript submitted]. Human PDPN-3, having two Tn-Thr residues, was further glycosylated with a recombinant T-synthase dC1Gal-T1 or a recombinant human core 3 synthase to produce the core 1 and core 3 (GlcNAcβ1-3GalNAcα1-O-Ser/Thr) glycoforms, respectively, as described previously [15]. The T-glycoform produced was shown by MALDI-TOF to be partially glycosylated with one and two T structures, while the core 3 glycoform mainly resulted in one core 3 structure (not shown). The PDPN-3-T and PDPN-3-C3 were included in the podoplanin glycopeptide library.

Table 1 Fragment ions obtained of the MH_5^{5+} precursor ion of pdpn-30mer-5Tn by ETD in the LTQ-Orbitrap. * marks fragments with low peak abundance. All masses are monoisotopic

Ion species	Calculated mass	Measured mass	Deviation (ppm)
MH_5^{5+}(mono)	863.4216	863.4237	2.4
z_4^+	507.1708	507.1718	2.0
z_5^+	594.2029	594.2040	1.9
z_6^+	695.2505	695.2532	3.9
z_7^+	766.2876	766.2899	3.0
z_8^+	1056.3990	1059.4008*	1.7
z_{17}^{2+}	1229.0493	1229.0494*	0.1
$z+1_{20}^{2+}$	1488.1780	1488.1792	0.8
$z+1_{21}^{2+}$	1552.6993	1552.7016	1.5
$z+1_{22}^{2+}$	1630.7499	1630.7530	1.9
$z+1_{23}^{2+}$	1694.7792	1694.7827	2.1
$z+1_{26}^{2+}$	1944.9033	1944.9042	0.5
c_3^+	314.2187	314.2194	2.2
c_4^+	427.3027	427.3035	1.9
c_6^+	623.4239	623.4250	1.8
c_7^+	927.5510	927.5530	2.2
c_8^+	1055.6095	1055.6120	2.4
c_9^+	1211.7106	1211.7138	2.6
c_9^{2+}	606.3590	606.3600	1.6
c_{10}^+	1340.7532	1340.7561	2.2
c_{10}^{2+}	670.8803	670.8816	1.9
c_{13}^+	1858.0029	1857.9918	6.0
c_{13}^{2+}	929.5051	929.5101*	5.4

Immunization of mice

The glycopeptide pdpn-30mer-3/5Tn was conjugated to KLH (Pierce, Rockford, IL) using glutaraldehyde and efficiency of the conjugation was evaluated by a comparative titration analysis of the KLH conjugate and glycopeptides in enzyme linked immunosorbent assay (ELISA). The analysis indicated that the conjugation was near complete, which should result in a KLH to glycopeptide ratio of 1:300. Female Balb/c mice were injected subcutaneously with 20 μg of glycopeptide-KLH in a total volume of 200 μL (1:1 mix with Freund's complete (first immunization only) and incomplete adjuvant, Sigma). Mice received 3 immunizations 14 days apart and blood samples were obtained by eye bleeding 1 week following the third immunization.

ELISA

96-well MaxiSorp plates (Nunc, Denmark) were coated over night at 4°C with 1 μg/ml of glycopeptide in a

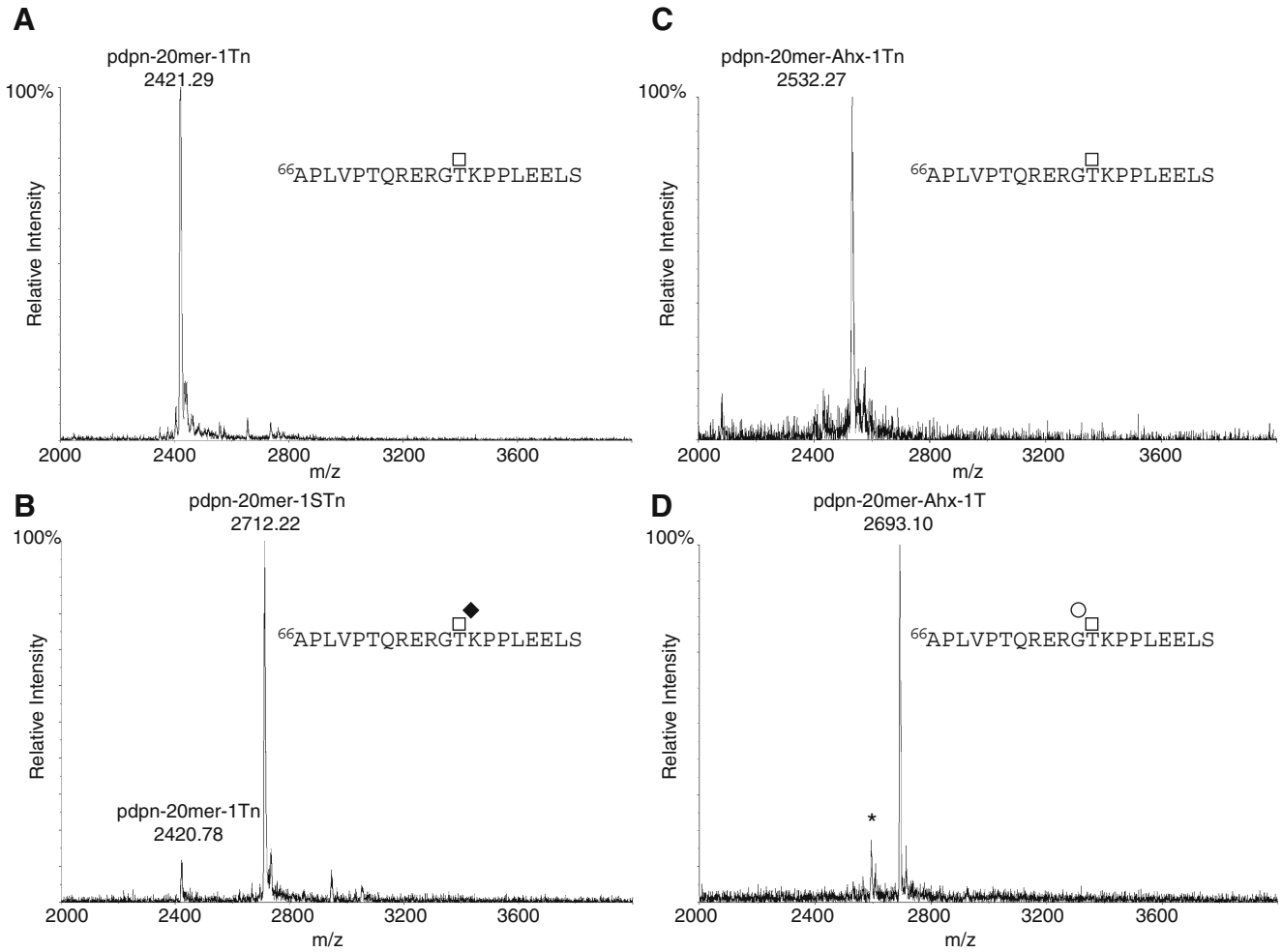


Fig. 4 Glycosylation of pdpn-20mer-1Tn. Pdpn-20mer-1Tn (**a**) was sialylated with ST6GalNAc-I. MALDI-TOF-MS analysis showed that sialylation was near complete (**b**) even though the sialic acid linkage is labile during ionization. The pdpn-20mer-1Tn was further more glycosylated with dCGal-T1 (**c**) resulting in complete conversion into the T-glycoform (**d**). The T-glycoform was made from pdpn-20mer-1Tn containing an aminohexanoic acid (Ahx) linker accounting for the mass difference of pdpn-20mer-1Tn in (**a**) and (**c**). *m/z 2584.98

$Na2CO_3$-buffer (pH 9.6). The plates were blocked in PLI-P buffer pH 7.4 (PO_4, Na/K, 1% Triton, 1% BSA) for 1 h at RT. Plates were then incubated with sera (diluted in PLI-P buffer) or MAb 5F4 [23], TKH2 [24], 3C9 [25] or Tn reactive HPA (Sigma) (1 μg/ml) for 1 h at RT. Bound antibodies were detected with HRP conjugated isotype and subclass specific goat anti-mouse antibodies (SouthernBiotech) applied in 1:2000 dilution. HPA binding was detected with streptavidin-HRP (1:4000) (Sigma). Plates were developed with TMB+ (Dako). Adding 0.5 M H_2SO_4 stopped the reaction and the plate was read at 450 nm. Serum from a mouse immunized with an irrelevant Tn-glycopeptide derived from the human IgA-hinge region with two GalNAc residues (VPSTPPTPSPSTPPTPSPSA) conjugated to KLH was included as a control. A glycopeptide based on human MUC-2 33mer (PTTTPITTTTTVTPTPTPTGTQTPTTT PISTTC-11Tn) was also included as a control.

Glycopeptide microarray analysis

The PDPN1-14, pdpn-30mer, pdpn-30mer-Tn, a naked MUC1 peptide, as well as two MUC1 Tn-glycopeptides were printed on Schott Nexterion® Slide H MPX 16 (Schott AG, Mainz, Germany). Quadruplicates of all compounds were printed at 1 mg/ml concentration in 150 mM sodium phosphate pH 8.5 with 0.005% CHAPS. Printing was performed on a BioRobotics MicroGrid II spotter (Genomics Solution) with a 0.21 mm pitch using Stealth 3B Micro Spotting Pins (Telechem International ArrayIt Division). After printing, slides were incubated for 1 h in a humidified hybridization chamber with 70–100% relative humidity. The slides were blocked for 1 h with 25 mM ethanolamine in 100 mM sodium borate pH 8.5. Commercially available sera from testis seminoma cancer (n=10) and squamous lung cell carcinoma (n=10) taken at time of

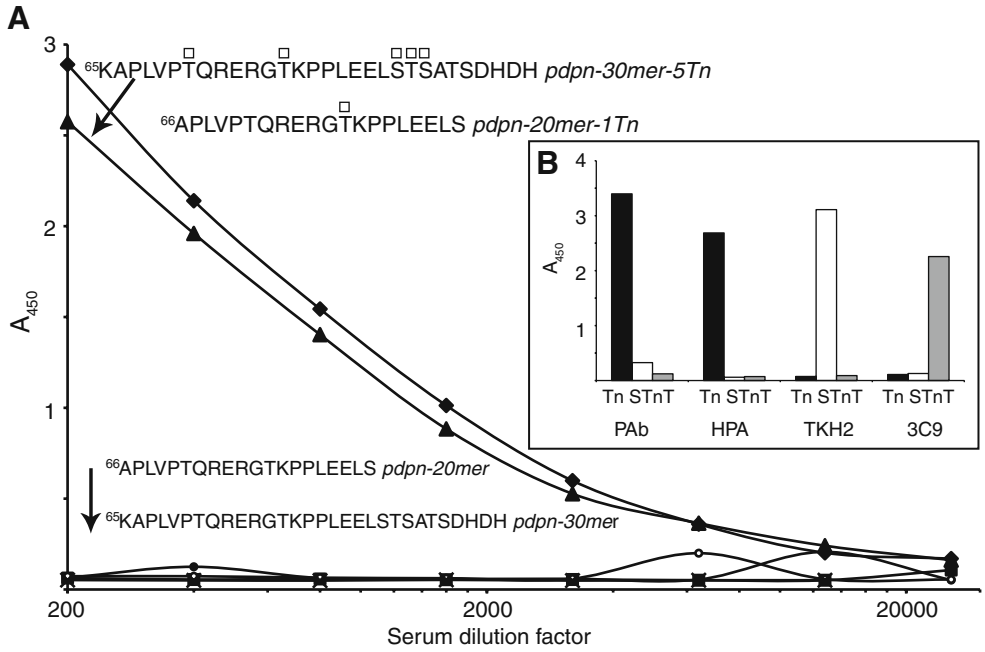


Fig. 5 Tn-glycopeptide specific immune response evaluated by ELISA. (**a**) ELISA analysis of the PAb response from one mice representative of the two mice immunized with pdpn-30mer-3/5Tn-KLH. Designations are as follows: ♦, pdpn-20mer-1Tn; ▲, pdpn-30mer-5Tn; ●, pdpn-30mer; ■, pdpn-20mer; ○, MUC2-11Tn; and ×, IgAh-2Tn. The serum contained a high IgG titer against pdpn-30mer-5Tn. A strong reactivity towards pdpn-20mer-1Tn showed that GalNAc on Thr^{77} was sufficient for epitope recognition. The PAb did not react with neither of the naked peptides (pdpn-30mer and pdpn-20mer) or other glycopeptides (MUC2-11Tn and IgA-hinge-2Tn). Serum from a mouse immunized with the glycopeptide covering the human IgA hinge region showed only reactivity to IgA-hinge-2Tn and not the other peptides (data not shown). (**b**) Sialylation of pdpn-20mer-1Tn resulted in abolishment of HPA reactivity and strong reactivity with MAb to STn TKH2 [24] in ELISA. The epitope of the PAb to pdpn-Tn was completely blocked by sialylation. The pdpn-20mer-1T glycopeptide was likewise only recognized by the T-binding MAb 3C9 [25] and not by HPA or the PAb to pdpn-Tn

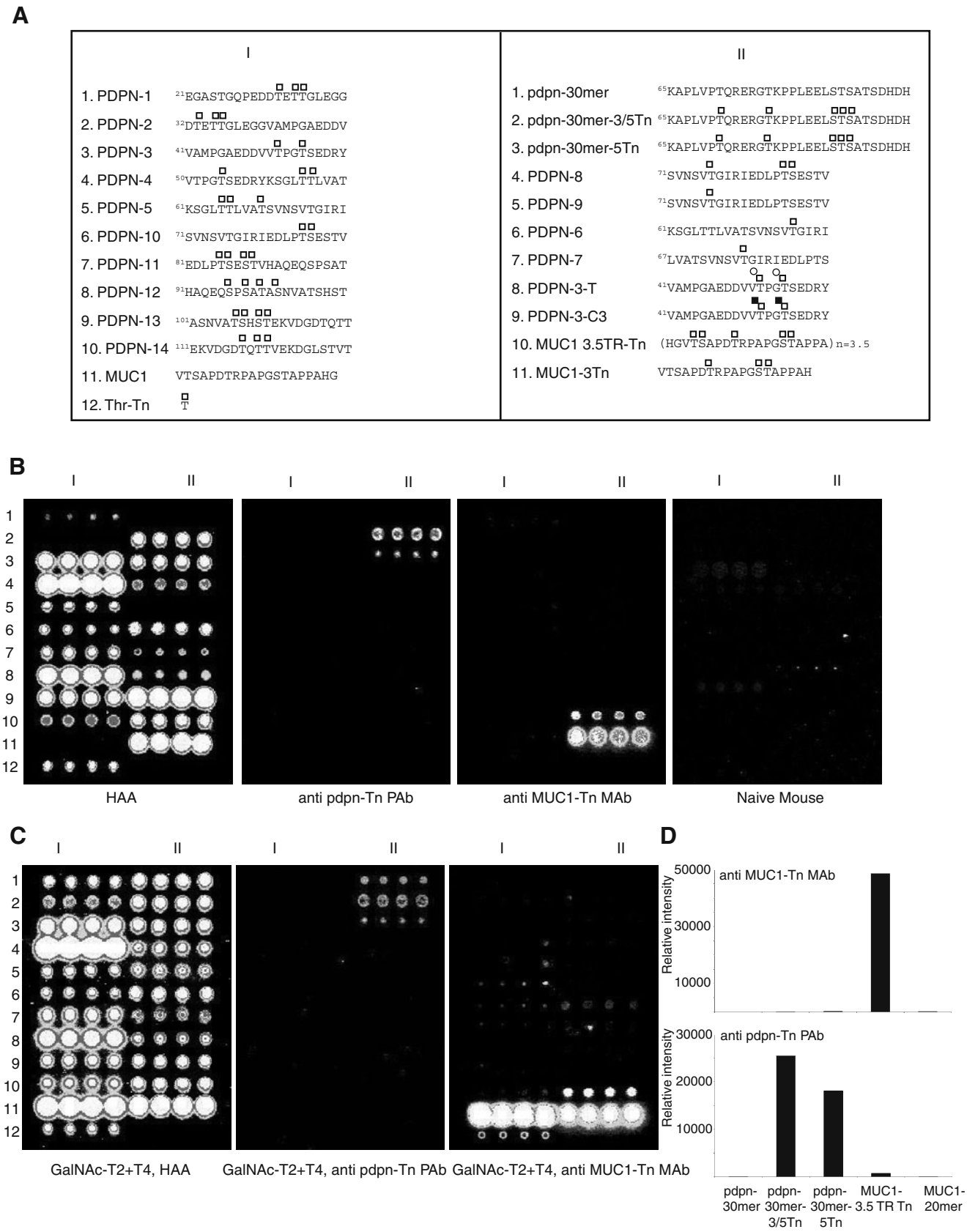
diagnosis were applied (Asterand). Sera (1:25, 1:50, 1:200 dilution), HPA (1 μg/ml), lectin from *Helix aspersa* (HAA) (1 μg/ml), MAb 5F4 [23] (undiluted hybridoma supernatant) and 5E5 [10] (diluted hybridoma supernatant 1:1600) were incubated on the slide for 1 h, followed by 1 h incubation with appropriate secondary antibodies. All dilutions were made in PLI-P buffer pH 7.4. Murine monoclonal antibodies were detected with Cy3-conjugated goat anti-mouse IgM and goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:4000. Human IgG antibodies were detected with Cy3-conjugated goat anti-human IgG (1:2000) and biotinlyated HPA (Sigma) detected with streptavidin-Alexa Fluor®488 (Invitrogen)(1:4000).

Glycosylation of printed (glyco)peptides on slides was performed after blocking by using purified recombinant human GalNAc-T2 and -T4 [O. Blixt *et al.* manuscript submitted]. Briefly the reaction mixture (50 μl/ well) contained 25 mM MES pH 7.4, 2 mM $MgCl_2$, 0.25% TX-100 and 200 μM UDP-GalNAc. Incubation was performed for 3 h at 37°C. All incubation steps were separated by two wash steps in PBS with 0.05% Tween-20 (PBS-T) and one in PBS. After the final wash, slides were rinsed in H_2O, dried by centrifugation (200×*g*) and scanned in a ProScanArray HT Microarray Scanner (PerkinElmer) followed by image analysis with ProScanArray Express 4.0 software (PerkinElmer).

Results

Synthesis and characterization of Tn and STn podoplanin glycopeptides

The study of Schietinger *et al.* [18] suggested that the immunodominant epitope for MAb237 included a Tn O-glycan at Thr^{77} in murine podoplanin. Since MAb 237 reacted strongly with podoplanin by reducing SDS-PAGE Western blotting, we predicted that the epitope would be continuous without conformational constraints. Therefore in order to design a glycopeptide vaccine covering the putative epitope, we used a synthetic 30mer peptide (pdpn-30mer, See Fig. 1) with Thr^{77} at position 13 and a total of 7 potential O-glycosylation sites. Glycosylation of this peptide with GalNAc-T2 resulted in a heterogeneous mixture of products carrying 3–5 GalNAc residues (pdpn-30mer-3/5Tn), while use of GalNAc-T4 alone resulted in glycosylation of a small subfraction of the peptide with 1–5 GalNAc residues (Fig. 2). The latter finding suggests that


A
I
1. PDPN-1 21EGASTGQPEDDTETTGLEGG
2. PDPN-2 32DTETTGLEGGVAMPGAEDDV
3. PDPN-3 41VAMPGAEDDVVTPGTSEDRY
4. PDPN-4 50VTPGTSEDRYKSGLTTLVAT
5. PDPN-5 61KSGLTTLVATSVNSVTGIRI
6. PDPN-10 71SVNSVTGIRIEDLPTSESTV
7. PDPN-11 81EDLPTSESTVHAQEQSPSAT
8. PDPN-12 91HAQEQSPSATASNVATSHST
9. PDPN-13 101ASNVATSHSTEKVDGDTQTT
10. PDPN-14 111EKVDGDTQTTVEKDGLSTVT
11. MUC1 VTSAPDTRPAPGSTAPPAHG
12. Thr-Tn T
II
1. pdpn-30mer 65KAPLVPTQRERGTKPPLEELSTSATSDHDH
2. pdpn-30mer-3/5Tn 65KAPLVPTQRERGTKPPLEELSTSATSDHDH
3. pdpn-30mer-5Tn 65KAPLVPTQRERGTKPPLEELSTSATSDHDH
4. PDPN-8 71SVNSVTGIRIEDLPTSESTV
5. PDPN-9 71SVNSVTGIRIEDLPTSESTV
6. PDPN-6 61KSGLTTLVATSVNSVTGIRI
7. PDPN-7 67LVATSVNSVTGIRIEDLPTS
8. PDPN-3-T 41VAMPGAEDDVVTPGTSEDRY
9. PDPN-3-C3 41VAMPGAEDDVVTPGTSEDRY
10. MUC1 3.5TR-Tn (HGVTSAPDTRPAPGSTAPPA)n=3.5
11. MUC1-3Tn VTSAPDTRPAPGSTAPPAH
B
I II I II I II I II
1 2 3 4 5 6 7 8 9 10 11 12
HAA
anti pdpn-Tn PAb
anti MUC1-Tn MAb
Naive Mouse
C
I II I II I II
1 2 3 4 5 6 7 8 9 10 11 12
GalNAc-T2+T4, HAA
GalNAc-T2+T4, anti pdpn-Tn PAb
GalNAc-T2+T4, anti MUC1-Tn MAb
D
anti MUC1-Tn MAb
Relative intensity
50000
30000
10000
anti pdpn-Tn PAb
Relative intensity
30000
20000
10000
pdpn-30mer
pdpn-30mer-3/5Tn
pdpn-30mer-5Tn
MUC1-3.5 TR Tn
MUC1-20mer

◀ **Fig. 6** Array analysis of the specificity of pdpn-Tn polyclonal immune response. Fourteen Tn-glycopeptides covering human podoplanin were synthesized and printed on an array together with pdpn-30mer and pdpn-30mer-Tn. As controls a MUC1 peptide as well as two MUC1-Tn peptides were included. The different glycopeptides presented in (**a**) were printed in quadruplicate in two columns, I and II. Printing was evaluated by HAA (**b**). Sera were added to the array in 1:200 dilution and reacted only with pdpn-30mer-Tn and none of the human glycopeptides. Sera from naive mice were added to the array (1:50) and did not show reactivity to any of the peptides. The MAb 5E5 (1:1600) specific for MUC1-Tn did not react with the murine or human glycopeptides but only with MUC1-Tn. On-slide glycosylation was performed with recombinant polypeptide GalNAcT-2 and T4 in combination (**c**). The reaction was validated with HAA. 5E5 reacted with the former naked MUC1 after on-slide glycosylation and the pdpn-Tn PAb did react with the former unglycosylated pdpn-30mer peptide. (**d**) A quantitative representation of the reactivity of MUC1 (1:1600) and the PAb response to pdpn-Tn (1:200). No cross-reactivity was observed with either of the antibodies. Sera from patients with testicular seminoma or squamous lung cell carcinoma were added to the array as well (1:25), but no auto-antibodies were detected to the Tn glycoform of human podoplanin (not shown)

GalNAc-T4 does not utilize this substrate without prior partial GalNAc-glycosylation, which could result from minor contaminating insect GalNAc-transferase activities in the GalNAc-T4 preparation [26, 27]. Reaction with a combination of GalNAc-T2 and -T4 resulted in an essentially homogenous glycopeptide with 5 GalNAc residues (pdpn-30mer-5Tn), and mass spectrometric analysis of sites of incorporation showed that $Thr^{7,13,22}$ and $Ser^{21,23}$ were glycosylated (Fig. 3 and Table 1), including the Thr residue at position 13 of the peptide equivalent to Thr^{77}. We also chemically synthesized two 20mers with and without a single GalNAc residue at position 12 (equivalent to Thr^{77} of podoplanin), pdpn-20mer and pdpn-20mer-1Tn, and further produced the STn glycoform (pdpn-20mer-1STn) and T glycoform (pdpn-20mer-1T) with the ST6GalNAc-I and dC1Gal-T1 enzymes respectively (Fig. 4).

The pdpn-30mer-3/5Tn glycopeptide elicited IgG antibodies specific for the glycopeptide

We chose to use the GalNAc-T2 glycosylated pdpn-30mer glycopeptide as immunogen to cover the greatest number of Tn-glycoforms possible. Mice immunized with pdpn-30mer-3/5Tn-KLH produced high titer IgG1 responses to the immunogen Tn-glycopeptide with no reactivity with the unglycosylated peptide or an irrelevant Tn-glycopeptide based on human MUC2 (Fig. 5a). The epitope was contained in the shorter pdpn-20mer-1Tn glycopeptide, confirming the location of the immunodominant epitope in the region around the single GalNAc residue at Thr^{77} in murine podoplanin. Since titration curves of the PAb on the single Tn-glycopeptide (pdpn-20mer-1Tn) and the Tn-glycopeptide with five GalNAc residues (pdpn-30mer-5Tn) were similar, it appears that the GalNAc residue at Thr^{77} is the major immunodominant epitope. To evaluate the glycoform specificity of the immune response we tested the STn and T glycoforms, pdpn-20mer-1STn and pdpn-20mer-1T. The PAb did not react with the chemoenzymatically produced STn and T glycoforms, indicating that the antibody was specific for the Tn glycoform (Fig. 5b). We have previously found that immunization with GalNAc-glycopeptides often results in polyclonal responses specific for the glycopeptide with no or little reactivity with the unglycosylated peptide or the Tn hapten epitope [9, 10].

Synthesis and analysis of O-glycopeptide microarrays

To further study the peptide specificity of the polyclonal response we used O-glycopeptide arrays as described previously [17]. The first array displayed, besides the (glyco)peptides covering the immunodominant epitope in murine podoplanin, an almost comprehensive library of 20mer GalNAc-glycopeptides covering the human podoplanin protein (PDPN) and irrelevant control peptides (Fig. 6a). The quality of synthesis and printing of the GalNAc-glycopeptides was confirmed by MALDI-TOF analysis and reactivity with Tn reactive lectins and monoclonal antibodies after immobilization on arrays (Fig. 6b). All compounds were immobilized at saturated printing conditions and almost all GalNAc-glycopeptides reacted with HAA. Although considerable variation in intensity was observed with this lectin, it indicates different binding efficiency of the lectin for Tn glycopeptides with different numbers and positions of GalNAc residues (unpublished observations). GalNAc-glycopeptides PDPN-2 and PDPN-9 did not react with HAA directly. On-slide glycosylation with GalNAc-T2 and -T4 enzymes enhanced the reactivity of HAA with several GalNAc-glycopeptides including the two that were previously unreactive (Fig. 6c). Since most of the GalNAc-glycopeptides have additional putative glycosylation sites, this would be consistent with incorporation of additional GalNAc residues. One inconsistency is that the PDPN-2 GalNAc-glycopeptide was reactive only after on-slide glycosylation with GalNAc-transferases, while there are no additional acceptor sites in the GalNAc-glycopeptide. However PDPN-2 did react directly with another lectin, HPA (not shown). Detection of GalNAc residues on glycopeptides immobilized through amino reactive groups may pose problems with certain peptide designs, such as those with GalNAc residues too close to the linker and glycopeptides with internal lysine residues that may compete for binding. Nevertheless, the on-slide glycosylation with GalNAc-T2 and -T4 was shown to induce lectin reactivity of the unglycosylated human MUC1 and pdpn-30mer peptides (Fig. 6b, column I, row 11 and column II, row 1, respectively). In agreement with this,

the on-slide glycosylation induced reactivity with the immunodominant epitope for the anti-pdpn-30mer-3/5Tn PAb as well as the mouse MAb 5E5 directed to a similar Tn-glycopeptide epitope in MUC1 [15] (Fig. 6c,d).

Murine and human podoplanin share general protein structure; however, the region covering the immunodominant Thr^{77} Tn-glycopeptide epitope is rather poorly conserved (Fig. 1). Nevertheless, we built and tested this library to potentially identify a cross-reactive epitope in human podoplanin. Interestingly, the PAb reacted only with the murine pdpn-Tn-glycopeptides and showed no reactivity with any of the human glycopeptides, indicating that the epitope defined by this PAb is highly restricted for the murine peptide sequence (Fig. 6b). Sera from unimmunized mice did not react with any glycopeptides on the array (Fig. 6b).

Finally, we also tested the PAb on a microarray displaying (glyco)peptides derived from human MUC1 and a series of recombinant mucin fragments from human MUC1, MUC2, MUC4, MUC5Ac, MUC6 and MUC7. The recombinant mucin fragments derived from tandem repeat regions were produced in E. coli as HIS tagged fusion peptides and purified by Ni-chromatography and glycosylated in solution with appropriate enzymes to achieve different O-glycosylation patterns [JW Pedersen *et al*. manuscript submitted]. The PAb elicited with pdpn-30mer-3/5Tn did not react with any compound on this array above background (not shown).

Discussion

In this study we provide further confirmation for the existence of distinct antibody epitopes comprised of both peptide and glycan moieties that essentially render these epitopes specific for glycosylation variants of a single protein. The podoplanin Tn-glycopeptide analyzed induced a potent polyclonal IgG1 antibody response with high specificity for the podoplanin peptide sequence and the Tn glycoform, and although the immunogen included multiple Tn glycans the minimum immunodominant epitope was confined to one Tn glycan at residue Thr^{77} in murine podoplanin. The results are in agreement with and extend the reported specificity of MAb 237 produced in syngeneic mice in response to the Ag104A tumor with a cosmc mutation [18]. The identified Tn-podoplanin glycopeptide epitope is therefore very similar to the immunodominant cancer-specific Tn-MUC1 epitope that we previously identified [10, 15]. Common to these epitopes are lack of immunological tolerance and the ability for spontaneous induction of auto-antibodies in response to cancer [15, 17].

The basis for immunity to aberrant truncated O-glycoproteins is poorly understood. Aberrant immature O-glycans such as Tn, T and STn are targeted by natural carbohydrate hapten antibodies mainly of IgM isotype, and the titers of these may increase in cancer [28]. We have shown that Tn-glycoforms of MUC1 are selectively targeted by dendritic cells and that Tn-glycopeptides are bound, internalized and localized to MHC class I and II compartments in dendritic cells [29]. Furthermore, truncated O-glycopeptides may constitute distinct T-cell epitopes [30, 31]. There are thus multiple avenues for stimulation of immunity to aberrantly O-glycosylated glycoproteins. Tn glycoforms of O-glycoproteins are natural biosynthetic intermediates occurring briefly in Golgi before further O-glycan elongation and capping in normal cells. The finding that some of these Tn-glycopeptide epitopes are not covered by immunological tolerance [10, 15, 18, 32] suggests that with respect to immunological self-tolerance these biosynthetic intermediates are not considered self molecules. This may relate to all truncated glycoforms normally only found in distinct Golgi compartments or to specific O-glycans for which there are innate lectin receptors, such as the macrophage C-type lectin specific for unsubstituted exposed Gal and GalNAc residues on glycoproteins [33, 34]. It is also possible that truncated O-glycans attached at sites in glycoproteins, that are not normally utilized, may participate in generation of auto-antibody epitopes. The initiation step of mucin-type O-glycosylation is under very complex regulation and up to twenty different polypeptide GalNAc-transferase isoforms, with partially different substrate specificiies, direct sites of O-glycan attachments in proteins [26, 35]. Since these isoforms exhibit marked changes in expression in cancer, it is conceivable that novel epitopes are generated by aberrant O-glycosylation in terms of both sites of O-glycan attachment and structures of the O-glycans at specific sites. Cancer cells almost always express Tn and T glycoforms of O-glycoproteins, and as demonstrated here cancer-induced immunity may be targeted specifically against aberrant glycoforms of glycoproteins. Thus, discovery programs aimed at identifying auto-antibodies with biomarker potential should clearly consider aberrant O-glycosylation and posttranslational modifications in general.

There is increasing evidence pointing to the significance of aberrantly posttranslational modified proteins for auto-immunity [36–38]. Currently there are only few examples of involvement of glycosylation. One important example is an immunodominant epitope in type II collagen comprising a glycosylated hydroxylysine residue that is involved in collagen-induced arthritis [39]. Glycosylation may also modulate protein processing and hence affect exposure of new epitopes as shown in Rasmussen's encephalitis, where an N-glycan blocks proteolysis of a neuronal glutamate receptor and the establishment of a short peptide epitope [40]. Several human monoclonal antibodies have been shown to be directed to epitopes affected by glycosylation [41–45] but it is currently unclear if these antibodies define glycopeptide

epitopes per se. The O-glycopeptide autoantibody epitopes identified on human MUC1 and murine podoplanin are therefore important models for this type of epitope.

Detection of combined aberrant O-glycopeptide epitope specific antibodies in human serum is not straightforward. Natural antibodies to the carbohydrate haptens may interfere or obscure detection by binding to the glycans alone, and these antibodies occur in healthy individuals as well as in various diseases. There are no simple ways to obtain distinct cancer glycoforms of O-glycoproteins and most high throughput screening platforms using recombinant proteins, phage displays and protein/peptide arrays either do not contain glycans or contain glycans different from those associated with cancer. As part of the specificity analysis we scanned the anti-murine Tn-podoplanin PAb against an almost complete 20mer Tn-glycopeptide library built on the human podoplanin sequence, which demonstrated that the immunodominant epitope was not conserved. Human and murine podoplanin only have limited sequence similarity and in particular the region with the Thr^{77} epitope is poorly conserved (Fig. 1). Preliminary analysis of human sera from cancer patients with testicular seminoma or squamous lung cell carcinoma did not detect any auto-antibodies on this array, but the array was limited to the Tn glycoform (not shown). Future studies should analyse glycopeptide arrays with more extended O-glycan structures, and with the chemoenzymatic synthesis developed using recombinant glycosyltransferases it should be feasible to produce more complex core structures with different termination such as fucosylation and sialylation.

In conclusion, the results confirm and extend the existence of a distinct Tn O-glycopeptide antibody epitope in murine podoplanin that is remarkably restricted in terms of both peptide sequence and glycoform. It may be predicted from these and our previous results that many similar cancer-specific truncated O-glycopeptide epitopes exist in other O-glycoproteins, which suggests that it is warranted to explore further the O-glycopeptide epitome for disease-associated auto-antibody targets.

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