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# Localisation of a Protein Core-specific Epitope from Gastrointestinal Mucin (MUC2). The Effect of Epitope Immobilisation on Antibody Recognition

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Abstract: Human intestinal mucins are high molecular weight glycoproteins which protect and lubricate the epithelium of the gastrointestinal tract. In cases of malignant disease, mucins are abnormally expressed, overproduced or underglycosylated. This feature may enable the mucins to serve as tumour markers. The MUC2 mucin largely consists of a variable number of tandem repeats of a 23 amino acid sequence, $^{1}$PTTTPITTTTTVTPTPTPTGTQT$^{23}$. In this study we have localised the minimal and the optimal epitope within this region by the previously developed protein core specific 996 monoclonal antibody using synthetic peptides. Several overlapping and truncated peptides related to the tandem repeat unit have been prepared by solid-phase methodology. Other mucin peptides were synthesised on the tips of polyethylene pins, and these remained C-terminally attached to the pins for comparative investigations. The interaction of the 996 monoclonal antibody with the synthetic peptides was studied either in solution by competition RIA or on immobilised peptides by indirect ELISA experiments. These experiments show that the minimal epitope recognised by the 996 antibody is the Ac-$^{19}$TGTQ$^{22}$ ($IC_{50} = 3100$ μM in solution). For the optimal 996 antibody binding in solution the $^{16}$PTPTGTQ$^{22}$ heptapeptide ($IC_{50} = 3$ μM) is required. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: MUC2 mucin; synthetic peptides; antibody recognition in solution; antibody recognition on immobilised peptides; B cell epitope of MUC2 mucin

## INTRODUCTION

Human intestinal mucins are high molecular weight glycoproteins (> 1000000 Da), which protect and lubricate the epithelium of the gastrointestinal tract [1]. Apart from these physiological functions, mucins are important because in the case of several diseases of the gastrointestinal tract they are overproduced or, for example with colonic cancer, abnormally glycosylated. Because of this feature mucins may have potential as tumour markers [2].

Several genes encoding mucins have now been identified [3–9]. The protein core of the gastrointes-

Abbreviations: ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonate); BSA, bovine serum albumin; DIC, Diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Ig, immunoglobulin; KLH, keyhole limpet haemocyanin; MUC2, gastrointestinal mucin glycoprotein; PBS, Phosphate buffered saline; RaMPS, rapid multiple peptide synthesis; RIA, radioimmunoassay.

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tinal mucin, defined by the MUC-2 gene, consists largely of multiple repeats of the 23 residue sequence $^{1}$PTTTPITTTTTVTPTPTPTGTQT$^{23}$ [4]. Oligosaccharide side-chains are in O-linkage to threonine residues in this sequence.

If glycosylation is defective or incomplete in mucins of the malignant phenotype, the protein core of the mucin glycoprotein will be more exposed to the environment (e.g. antibodies). It follows that one strategy for the induction of antibody responses against tumour related mucin would be to immunise with deglycosylated mucin or with synthetic peptides corresponding to the sequences of immunodominant epitope(s). This latter approach was adopted for the induction of anti-MUC2 protein core monoclonal antibodies with potentially preferential reactivity with MUC2 mucin of the malignant phenotype. Based on prediction analysis, a synthetic peptide comprising the hydrophilic $^{12}$VTPTPTPTGTQTPT$^{25}$ region of the MUC2 was prepared with an additional Lys on its N-terminus (K$^{12}$VTPTPTPTGTQTPT$^{25}$-OH) [10]. This peptide was conjugated to keyhole limpet haemocyanin (KLH) for the induction of polypeptide core-specific monoclonal antibodies. Monoclonal antibody 996 (complete designation: 996/1/45/10 $IgG_1$) was produced which was shown to be reactive with both colorectal, gastric and ovarian carcinoma cell lines as well as primary human tumour tissues [11].

For the localization of the epitope recognised by the 996 antibody within the $^{1}$PTTTPITTTTTV-TPTPTPTGTQT$^{23}$ tandem repeat region, overlapping heptapeptides immobilised on polyethylene pins were produced. In Pepscan analysis [13] using the 996 monoclonal antibody and these heptamers a tetrapeptide sequence ($^{19}$TGTQ$^{22}$) was deduced as the functional epitope [14]. In preliminary experiments it was demonstrated that a longer peptide, $^{19}$TGTQTPTT$^{26}$-OH containing the deduced core epitope did not interact with the 996 antibody in solution.

Based on this observation we aimed (a) to determine the minimal and optimal epitope recognised by the 996 monoclonal antibody in solution, and (b) to define structural requirements of antibody recognition of this MUC2 epitope in solution and in its solid matrix immobilised form. To achieve this we have prepared several peptides containing the $^{19}$TGTQ$^{22}$ sequence, and their antibody binding was analysed. Peptides were assembled by solid-phase peptide synthesis and, after removal from the solid support, their recognition by antibody was tested in competition RIA assays in solution. Another group of peptides was synthesised on the tips of polyethylene pins and the antibody binding properties of this latter group was studied in ELISA assays. The binding properties of solid-phase-tethered and free peptides were compared.

## MATERIALS AND METHODS

### Peptide Synthesis and Purification

The peptides were prepared on a Multiple Peptide Synthesis System (RaMPS, DuPont Medical Product Department, E.I. du Pont de Nemours, Boston, MA) by solid-phase methodology using *p*-benzyloxy-benzylalcohol resin (Wang resin) [15] for free carboxy terminus peptides and 2,4-dimethoxy-benzhydrylamine resin [16] (Rapidamide, DuPont Medical Product Department, E.I. du Pont de Nemours, Boston, MA) for peptide amides, respectively. All amino acids were coupled as Fmoc-derivatives. The *tert*-butyl group was applied as a protecting group for the side-chain of Thr. Coupling was carried out using pentafluorophenyl or 3-hydroxy-4-oxo-3,4-dihydrobenzotriazine active esters or HOBt/DIC methodology in DMF. Fmoc groups were removed by 50% piperidine in DMF. The success of the coupling and deprotection steps was monitored by the ninhydrin test [17] and/or bromophenol blue assay [18]. *N*-acetylated peptides were prepared by acetic anhydride–diisopropylethylamine–DMF 5:1:50 (v/v/v). After the completion of the synthesis the peptides were cleaved from the resin with TFA containing 5% water. The peptides were purified by reverse phase HPLC on a DELTA-PAK column (7.8 mm × 30 cm, $C_{18}$-300Å, 15 μm, Spherical, Nihon Waters, Tokyo, Japan) using gradient elution, where eluent A: 0.1% TFA in water, while eluent B: 0.1% TFA in acetonitrile–water (80:20, v/v). After sample application isocratic elution with 0 or 10% B was applied for 5 min, a linear gradient from 0% B to 25% B or 10% B to 35% B was generated and applied over 25 min at room temperature with a flow rate of 1 ml/min. The amino acid composition of the peptides was verified by amino acid analysis. The correct relative molecular mass was determined by plasma desorption or electronspray mass spectrometry (Applied Biosystems BIO-ION 2 or VG-ZA-2SEQ, Fisons, Loughborough, UK).

### Peptide Synthesis on Polyethylene Pins

Peptides were prepared on the tips of polyethylene pins by solid-phase methodology using Fmoc chem-

istry. The coupling reaction was performed by pentafluorophenyl or 3-hydroxy-4-oxo-3,4-dihydrobenzotriazine active ester in DMF. The Fmoc groups were removed by 20% piperidine in DMF. After the completion of the synthesis, the amino terminus of the peptides was acetylated with a mixture of DMF–acetic anhydride–diisopropylethylamine 50:5:1 (v/v/v) for 90 min at RT. Then the side-chain protecting groups were removed with TFA containing 2.5% EDT, 2.5% phenol and 2.5% thioanisole, 4 h. The peptides were prepared in duplicates.

### Conjugation of Peptides to Bovine Serum Albumin (BSA)

Eight milligrammes BSA and 5 mg peptide amide dissolved in 3 ml 0.1 M $NaHCO_3$, pH 8.4 were mixed and 10 μl 25% glutaraldehyde (Grade 1, Sigma, Poole, UK) was added. After incubation for 3 h at room temperature, the product was dialysed overnight against 0.15 M NaCl at 4°C and then filtered through a 0.2 μm filter before storage at −20°C.

### ELISA Procedure using Peptide–BSA Conjugates as Target Antigens

The ability of the 996 antibody to bind peptide–BSA conjugates was measured using an ELISA assay. Conjugates at concentrations 10 and 1 μg/ml in PBS were dispensed at 100 μl per well into 3912 FALCON III flexible PVC plate (Becton Dickinson, Oxnard, CA) and dried at 37°C overnight. The wells were washed four times with PBS containing 1% BSA, and incubation was continued for 1 h at room temperature to block any remaining non-specific adsorption sites. The microtitre plate was washed with PBS containing 0.1% Tween 20. Monoclonal antibody 996 was added at different concentrations (0.1, 1, 10 μg/ml in PBS containing 0.1% BSA) in triplicates at 100 μl/well. After incubation for 2 h the antibody solution was removed and the wells were washed four times with PBS/Tween 20 washing buffer. Horse radish peroxidase (HPO)-linked rabbit anti-mouse Ig (Dako, High Wycombe, UK) at a dilution of 1/500 (in PBS containing 1% BSA) was dispensed at 50 μl/well, incubated for 1 h at room temperature, and the plate was again thoroughly washed. The ABTS substrate solution (50 mg 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonate in 100 ml of 0.1 M citrate–phosphate buffer, pH 4.0, containing 0.01% hydrogen peroxide) was added at 150 μl/well. $V_{max}$ measurements of absorbance at 405 nm were made for reactions in each well using Milenia Kinetic Analyzer (Diagnostic Products, CA, USA).

### Inhibition Radioisotopic Immunoglobulin Assay

Synthetic peptides in solution were further tested for their capacity to inhibit 996 antibody binding to [$K^{12}$VTPTPTPTGTQTPT$^{25}$-OH]–BSA conjugate. Conjugate at 1 μg/ml in PBS containing 0.02% $NaN_3$ was added to 60-well Terasaki Microtest Plates (well capacity = 10 μl; Nunc, Roskilde, Denmark) at 10 μl/well. Antigen containing plates were air dried. The wells were washed four times with a washing buffer consisting of PBS containing 0.1% casein and 0.02% $NaN_3$. During the final wash cycle, the wells were incubated with washing buffer for at least 30 min to complete the blocking of non-specific adsorption sites. $^{125}$I-labelled 996 antibody was prepared by the chloramine T procedure [12] with 18 MBq $^{125}$I for 25 μg protein. $^{125}$I-labelled 996 antibody at 1 μg/ml concentration, $2.8 \times 10^4$–$2.8 \times 10^5$ cpm/5 μl/well or washing buffer alone in negative controls was added to the wells to which 5 μl of the test peptide solution had been added (in a concentration range between 25 and 0.0003 mmol/l). After incubation for 2 h at room temperature the wells were aspirated and washed six times. The wells were finally separated and counted individually in an LKB Compugamma Counter (Stockholm, Sweden). In each case, the amount of added peptide required to inhibit antibody binding by 50% ($IC_{50}$) was calculated as a measure of antigenic potency.

### ELISA Procedure on Peptides Immobilised on Polyethylene Pins

The pins with immobilised test peptides were submerged into a blocking solution containing 1% ovalbumin, 1% bovine serum albumin, 0.1% Tween 20 and 0.05% $NaN_3$ in PBS for 1 h to block the non-specific adsorption sites. The antibody solution was added, 10 μg/ml, 175 μl/well, incubated overnight at 4°C. After washing four times in PBS/Tween 20 washing buffer, the pins were exposed to the 1/1000 dilution of HPO conjugated rabbit anti mouse immunoglobulin (175 μl/well) for 1 h at RT. After washing the pins (four times) tetramethylbenzydine was added (Cozart Bioscience, Abingdon, UK), at 150 μl/well. Absorbance at 605 nm was recorded in each well using Milenia Kinetic Analyzer (Diagnostic Products, CA, USA).

Table 1 Characteristic data of MUC2 peptides

| Peptide | Amino acid composition | $R_t$[a] | Relative molecular mass | |
|---|---|---|---|---|
| | Found (calculated) | (min) | Calc | $MH^+$obs |
| $^{13}$TPTPTPTGTQTPTT$^{26}$-$NH_2$ | T 7.40 (8), P 4.09 (4), G 1.33 (1), Q 1.26 (1) | 13.3[b] | 1398.7 | 1400.0[e] |
| $^{13}$TPTPTPTGTQT$^{23}$-OH | T 5.47 (6), P 2.80 (3), G 1.09 (1), Q 1.09 (1) | 19.3[c] | 1100.5 | 1101.8[e] |
| $^{13}$TPTPTPTG$^{20}$-OH | T 3.90 (4), P 3.07 (3), G 1.03 (1) | 7.8[b] | 770.4 | 772.1[e] |
| $^{13}$TPTPT$^{17}$-OH | T 2.87 (3), P 2.12 (2) | 20.1[d] | 515.3 | 517.0[f] |
| $^{15}$TPTPTGTQ$^{22}$-OH | T 3.77 (4), P 2.20 (2), G 1.00 (1), Q 1.03 (1) | 11.5[c] | 801.4 | 801.5[f] |
| $^{16}$PTPTGTQTPTT$^{26}$-OH | T 5.82 (6), P 3.00 (3), G 1.05 (1), Q 1.13 (1) | 11.4[b] | 1100.5 | 1101.9[f] |
| $^{16}$TQTPTT$^{26}$-$NH_2$ | T 6.03 (6), P 2.74 (3), G 1.04 (1), Q 1.18 (1) | 18.9[d] | 1099.5 | 1100.6[e] |
| $^{16}$PTPTGTQ$^{22}$-OH | T 2.80 (3), P 2.13 (2), G 0.97 (1), Q 1.02 (1) | 7.6[c] | 700.3 | 700.4[f] |
| $^{17}$TPTGTQ$^{22}$-OH | T 2.77 (3), P 1.28 (1), G 0.97 (1), Q 0.98 (1) | 13.0[d] | 603.3 | 604.6[e] |
| Ac-$^{18}$PTGTQ$^{22}$-OH | T 1.87 (2), P 0.95 (1), G 1.09 (1), Q 1.08 (1) | 17.2[d] | 544.3 | 545.3[c] |
| Ac-$^{18}$PTGTQ$^{22}$-$NH_2$ | T 1.87 (2), P 0.94 (1), G 1.09 (1), Q 1.10 (1) | 16.6[d] | 543.3 | 544.3[e] |
| $^{18}$PTGTQ$^{22}$-OH | T 1.98 (2), P 1.01 (1), G O.98 (1), Q 1.04 (1) | 6.8[d] | 502.2 | 503.3[e] |
| $^{18}$PTGTQ$^{22}$-$NH_2$ | T 1.82 (2), P 1.00 (1), G 1.12 (1), Q 1.05 (1) | 4.6[d] | 501.3 | 502.3[e] |
| $^{19}$TGTQTPTT$^{26}$-OH | T 4.82 (5), P 1.02 (1), G 1.04 (1), Q 1.14 (1) | 13.6[c] | 805.4 | 806.2[f] |
| $^{19}$TGTQTPTT$^{26}$-$NH_2$ | T 5.07 (5), P 0.88 (1), G 0.99 (1), Q 1.05 (1) | 5.5[d] | 804.4 | 804.4[e] |
| $^{19}$TGTQT$^{23}$-OH | T 2.89 (3), G 1.00 (1), Q 1.11 (1) | 8.9[d] | 506.3 | n.d.[g] |
| Ac-$^{19}$TGTQ$^{22}$-OH | T 1.84 (2), G 1.09 (1), Q 1.08 (1) | 7.3[d] | 447.2 | 448.2[e] |
| Ac-$^{19}$TGTQ$^{22}$-$NH_2$ | T 1.85 (2), G 1.10 (1), Q 1.06 (1) | 5.7[d] | 446.3 | 447.3[e] |
| $^{19}$TGTQ$^{22}$-OH | T 1.97 (2), G 0.98 (1), Q 1.05 (1) | 4.1[d] | 405.2 | 406.7[e] |
| $^{19}$TGTQ$^{22}$-$NH_2$ | T 1.84 (2), G 1.08 (1), Q 1.08 (1) | 3.7[d] | 404.2 | 405.2[e] |
| $^{22}$QTPTT$^{26}$-OH | T 2.85 (3), P 1.01 (1), Q 1.12 (1) | 7.0[c] | 546.3 | 547.4[f] |

HPLC was performed on DELTA-PAK 300Å, 15 μ, 3.9 mm × 300 mm column, with 1 ml/min flow rate, detection at 220 nm. Eluents: A: O. 1% TFA/water, B: 0.1% TFA/acetonitrile/water (80:20 v/v). Relative molar mass was determined by mass spectrometry.
[a] HPLC retention time; [b] 0–5 min 10% B eluent, 5–30 min 10–30% B eluent; [c] 0–5 min 5% B eluent, 5–30 min 5–30% B eluent; [d] 0–5 min 1% B eluent, 5–30 min 1–25% B eluent;[e] PD-MS; [f] FAB-MS; [g] no data.

## RESULTS

### Peptide Synthesis and Purification

We have synthesised 17 peptides and peptide amides with free $\alpha$-amino group corresponding to parts of the $^{13}$TPTPTPTGTQTPTT$^{26}$ region of the gastrointestinal mucin (MUC2) glycoprotein. In a few cases $N^{\alpha}$-acetyl derivatives of peptides were also prepared (Table 1). The amino acid analysis and the mass spectrometry show the expected primary structure. According to the HPLC chromatograms after purification, the purity of the peptides is greater than 95%. Characteristic data of the peptides including HPLC retention times, relative molecular mass and amino acid composition values are shown in Table 1.

Further 8 peptides containing the $^{19}$TGTQ$^{22}$ motif, and elongated either on the C- or N-terminus were prepared on polyethylene pins.

### Binding of 996 Monoclonal Antibody to BSA–Peptide Amide Conjugates

A preliminary ELISA assay performed with the 996 monoclonal antibody on BSA-conjugated MUC2 peptides as target antigen indicated that the antibody recognised the BSA-[$^{13}$TPTPTPTGTQTPTT$^{26}$-$NH_2$] and BSA-[$^{16}$PTPTGTQTPTT$^{26}NH_2$] conjugates, but it failed to bind the BSA-conjugate of the $^{19}$TGTQTPTT$^{26}$-$NH_2$ peptide (Table 2).

### Binding of 996 Monoclonal Antibody to MUC2 Peptides in Solution

In the RIA inhibition experiments we have investigated the $^{13}$TPTPTPTGTQTPTT$^{26}$-$NH_2$ peptide and its N-terminally truncated derivatives. Figure 1 shows that $^{13}$TPTPTPTGTQTPTT$^{26}$-$NH_2$ and $^{16}$PTPTGTQTPTT$^{26}$-OH peptides inhibit the binding of 996 monoclonal antibody to the BSA-[K$^{12}$VTPTPTPTGTQTPT$^{25}$-OH] target antigen with

Table 2 Binding of 996 antibody to BSA–peptide amide conjugate target antigens assayed by ELISA

| Concentration of conjugates | Antibody concentration | | |
|---|---|---|---|
| | 10 μg/ml | 1 μg/ml | 0.1 μg/ml |
| (μg/ml) | Binding (mOD/min)[a] | | |
| BSA–[$^{13}$TPTPTPTGTQTPTT$^{26}$-$NH_2$] | | | |
| 10 | 553.3 ± 21.7 | 439.6 ± 26.2 | 94.8 ± 7.1 |
| 1 | 101.0 ± 7.5 | 49.1 ± 0.6 | 6.5 ± 0.0 |
| BSA–[$^{16}$PTPTGTQTPTT$^{26}$-$NH_2$] | | | |
| 10 | 234.2 ± 8.2 | 127.9 ± 4.4 | 32.2 ± 1.4 |
| 1 | 29.0 ± 8.3 | 7.7 ± 5.9 | 2.1 ± 0.9 |
| BSA–[$^{19}$TGTQTPTT$^{26}$-$NH_2$] | | | |
| 10 | 11.4 ± 0.1 | 4.1 ± 5.6 | 21.4 ± 7.0 |
| 1 | −4.0 ± 7.1 | 4.8 ± 2.8 | 23.2 ± 8.3 |

[a] Optical density (OD) was measured at $\lambda = 405$ nm.

$IC_{50}$ values of 8.2 and 1.3 μM, respectively, while the peptides $^{19}$TGTQTPTT$^{26}$-OH and $^{22}$QTPTT$^{26}$-OH (data not shown) have no interaction with the antibody (Figure 1).

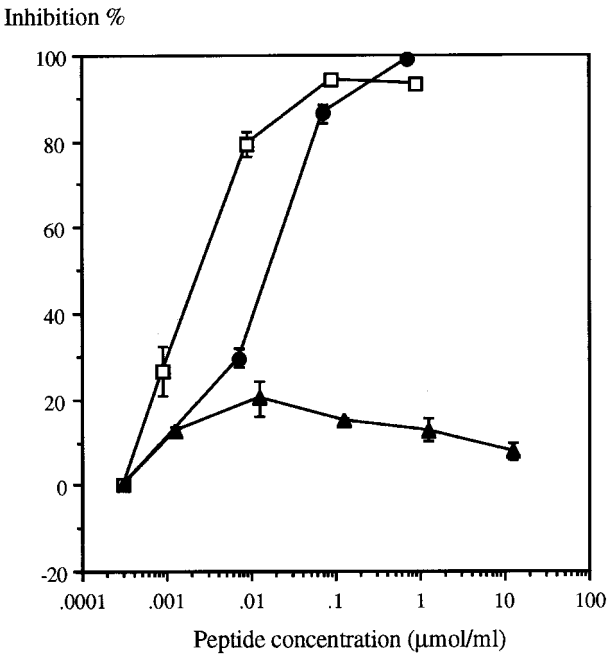


Figure 1 Inhibition of 996 antibody binding to BSA-[K$^{12}$VTPTPTPTGTQTPT$^{25}$-OH] target antigen with peptide $^{13}$TPTPTPTGTQTPIT$^{26}$-$NH_2$ (●), $^{16}$PTPTGTQTPTT$^{26}$OH (□) and $^{19}$TGTQTPTT$^{26}$-OH (▲) in a RIA competition assay.

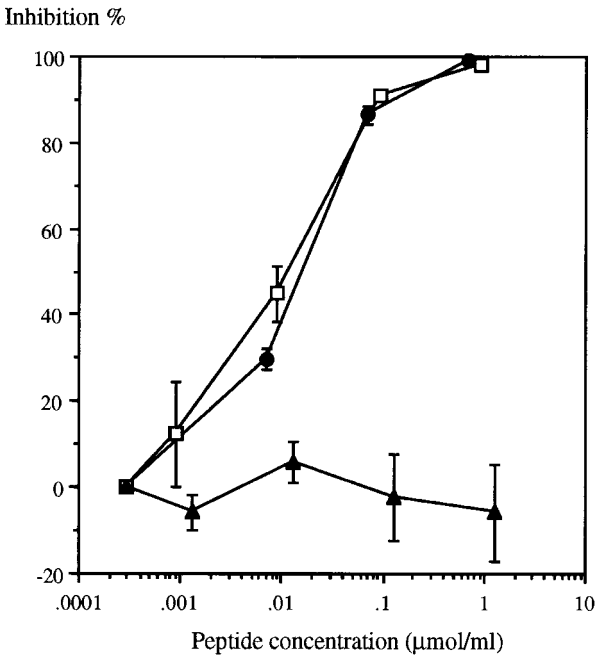


Figure 2 Inhibition of 996 antibody binding to BSA-[K$^{12}$VTPTPTPTGTQTPT$^{25}$-OH] target antigen with peptide $^{13}$TPTPTPTGTQTPTT$^{26}$-$NH_2$ (●), $^{13}$TPTPTPTGTQT$^{23}$-OH (□) and $^{13}$TPTPTPTG$^{20}$-OH (▲) in a RIA competition assay.

In the second series of RIA experiments, peptides shortened at the C-terminus were studied (Figure 2). The $^{13}$TPTPTPTGTQT$^{23}$-OH peptide, like $^{13}$TPTPTPTGTQTPTT$^{26}$-$NH_2$, inhibited the antibody binding ($IC_{50} = 5.9$ μM), but the shorter peptides, $^{13}$TPTPTPTG$^{20}$-OH and $^{13}$TPTPT$^{17}$-OH (data not shown), had no effect on antibody–target antigen interaction.

Results with N- or C-terminally truncated peptides suggest that the epitope recognised by the 996 monoclonal antibody is located within the $^{16}$PTPTGTQT$^{23}$ region.

A further series of peptides corresponding to the $^{16}$PTPTGTQT$^{23}$ sequence were prepared and investigated by inhibition RIA assay. Figure 3 shows that peptide $^{18}$PTGTQ$^{22}$-OH is the smallest peptide capable to inhibit the 996 antibody binding in solution ($IC_{50} = 62.2$ μM). Peptide $^{17}$TPTGTQ$^{22}$-OH–antibody interaction is stronger by one order of magnitude than that of the $^{18}$PTGTQ$^{22}$-OH ($IC_{50} = 7.0$ μM). The peptide concentrations at 50% inhibition for peptides $^{16}$PTPTGTQ$^{22}$-OH and $^{15}$TPTPTGTQ$^{22}$-OH are very low ($IC_{50} = 3.0$ and 2.8 μM, respectively) indicating the pronounced binding. Peptides shorter than $^{18}$PTGTQ$^{22}$-OH, like $^{19}$TGTQ$^{22}$-OH and

$^{19}$TGTQT$^{23}$-OH did not inhibit the 996 antibody binding.

The inhibitory capacity of *N*-acetylated peptides and peptide amides were also studied. Figure 4. shows that Ac-$^{19}$TGTQ$^{22}$-OH and Ac-$^{19}$TGTQ$^{22}$-$NH_2$ are equally capable to inhibit antibody binding but only at high concentration ($IC_{50} = 3100$ μM). $^{19}$TGTQ$^{22}$-$NH_2$ (Figure 4) or $^{19}$TGTQ$^{22}$-OH (data not shown) with free $NH_2$-group did not bind to the antibody. The inhibitory potential of peptide Ac-$^{18}$PTGTQ$^{22}$-$NH_2$ ($IC_{50} = 62$ μM) was essentially the same as that of $^{18}$PTGTQ$^{22}$-OH ($IC_{50} = 62.2$ μM), while $^{18}$PTGTQ$^{22}$-$NH_2$ was recognised somewhat less efficiently ($IC_{50} = 310$ μM).

### Binding of 996 Monoclonal Antibody to MUC2 Peptides Immobilised on Polyethylene Pins

In the next set of ELISA experiments C-terminally pin-attached peptides corresponding to the $^{16}$PTPTGTQT$^{23}$ sequence were investigated. As Figure 5 shows the 996 monoclonal antibody interacted strongly with peptides Ac-$^{16}$PTPTGTQ$^{22}$-pin, Ac-$^{17}$TPTGTQ$^{22}$-pin and Ac-$^{18}$PTGTQ$^{22}$-pin immobilised on the pins. The recognition of Ac-$^{19}$TGTQ$^{22}$-pin by monoclonal antibody 996 was significantly, ten times less efficient. In the case of $^{19}$TGTQ$^{22}$-pin no binding was observed.

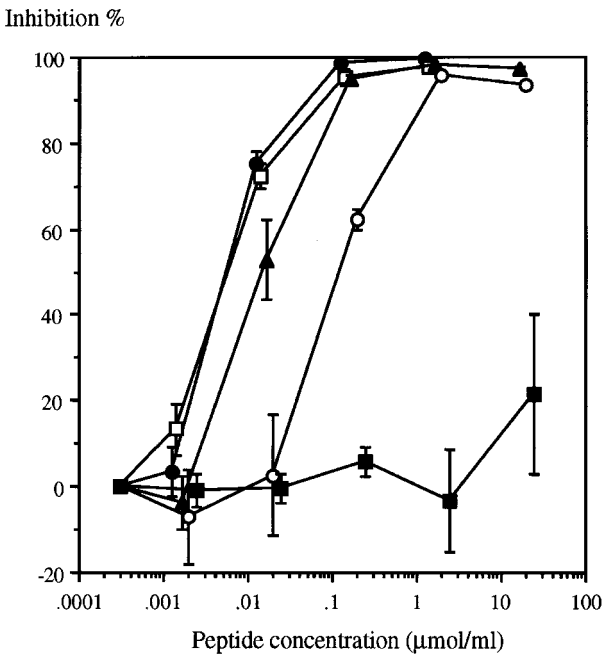


Figure 3 Inhibition of 996 antibody binding to BSA-[K$^{12}$VTPTPTPTGTQTPT$^{25}$-OH] target antigen with peptide $^{15}$TPTPTGTQ$^{22}$-OH (●), $^{16}$PTPTGTQ$^{22}$-OH (□), $^{17}$TPTGTQ$^{22}$-OH (▲), $^{18}$PTGTQ$^{22}$-OH (○) and $^{19}$TGTQ$^{22}$-OH (■) in a RIA competition assay.

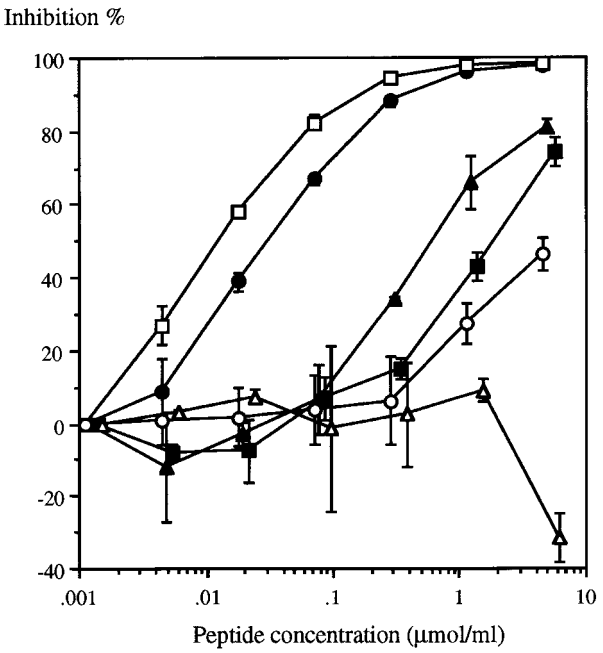


Figure 4 Inhibition of 996 antibody binding to BSA-[K$^{12}$VTPTPTPTGTQTPT$^{25}$-OH] target antigen with peptide Ac-$^{18}$PTGTQ$^{22}$-$NH_2$ (●), Ac-$^{18}$PTGTQ$^{22}$-OH (□), $^{18}$PTGTQ$^{22}$-$NH_2$ (▲), Ac-$^{19}$TGTQ$^{22}$-$NH_2$ (○), Ac-$^{19}$TGTQ$^{22}$-OH (■) and $^{19}$TGTQ$^{22}$-$NH_2$ (△) in a RIA competition assay.

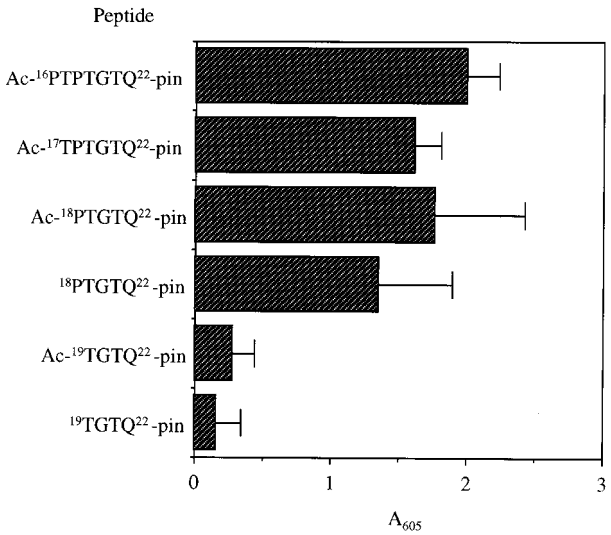


Figure 5 Binding of 996 antibody to MUC2 peptides immobilised to polyethylene pins, assayed by ELISA. Absorbance measured at 605 nm.

## DISCUSSION

MAb 996 was raised against a synthetic peptide ($K^{12}$VTPTPTPTGTTPT$^{25}$-OH) with a sequence based upon that of the hydrophilic region of the MUC2 mucin tandem repeat region [11]. This antibody also reacts with several cell lines derived from human gastrointestinal tumour tissues. From Pepscan data using overlapping heptamers and monoclonal antibody 996, an antibody epitope was located to the $^{19}$TGTQ$^{22}$ sequence [14].

In this study several peptides related to the $^{13}$TPTPTPTGTQTPTT$^{26}$ region of the tandem repeat unit of the MUC2 glycoprotein have been prepared and characterised. The binding of these peptides to the MUC2 peptide specific monoclonal antibody 996 has been investigated under various conditions. Free peptides in solution in competition RIA assays or peptides immobilised on polyethylene pins were reacted with MAb 996. Peptides conjugated to BSA and adsorbed to ELISA plates were also studied.

In the first set of experiments BSA-conjugates of peptides containing the deduced epitope $^{19}$TGTQ$^{22}$ were analysed by ELISA. Interestingly, 996 monoclonal antibody binding was observed to peptides in which $^{19}$TGTQ$^{22}$ was elongated by both N- and C-terminal residues. It should be noted that all three peptides studied were attached to BSA at their N-terminal amino acid residue. Therefore the lack of specific interaction between [$^{19}$TGTQTPTT$^{26}$-$NH_2$]–BSA containing the $^{19}$TGTQ$^{22}$ motif and MAb 996 could be attributed to the sterically hindered arrangement of the deduced epitope.

The RIA competition assay performed with $^{13}$TPTPTPTGTQTPTT$^{26}$-$NH_2$ and N-terminally truncated peptides indicated that the presence of N-terminal residue(s) (e.g. $^{16}$PTP$^{18}$) of $^{19}$TGTQ$^{22}$-OH is essential for antibody binding. Studies with peptides shortened at the C-terminus suggested that the removal of the three residues ($^{23}$PTT$^{26}$) had no influence on antibody binding, but the lack of $^{21}$TQ$^{22}$ from the deduced epitope resulted in complete loss of recognition. From this observation we concluded that the contribution of C-terminal residues situated outside of the deduced epitope to the antibody–epitope interaction is negligible.

The above conclusions were supported by another set of RIA results which show that the smallest peptide recognised by the 996 monoclonal antibody in solution is the Ac-$^{19}$TGTQ$^{22}$-OH peptide. However, the 996 binding to this peptide is weak. The antibody binds an order of magnitude more strongly to the $^{18}$PTGTQ$^{22}$-OH peptide, and the recognition proved to be highly efficient in case of the $^{16}$PTPTGTQ$^{22}$-OH peptide. Based on these results it is reasonable to conclude that the $^{19}$TGTQ$^{22}$ tetrapeptide representing the deduced B-cell epitope of the $^{13}$TPTPTPTGTQTPTT$^{26}$ region is needed for MAb 996 recognition. However, for specific interaction there is a need for an additional amide bond provided by acetylation or Pro at position 18 at the N-terminus. Therefore peptide Ac-$^{19}$TGTQ$^{22}$-OH can be considered as a minimal size functional epitope. Further modification of this structure by incorporation of $^{16}$PTP$^{18}$ resulted in a peptide whose 996 monoclonal antibody recognition in solution could be defined as optimal.

In order to study the structural characteristics of solid matrix immobilised epitope recognition ELISA experiments were performed on peptides attached to polyethylene pins. For this analysis peptides corresponding to the $^{16}$PTPTGTQ$^{22}$ sequence were designed. The deduced epitope ($^{19}$TGTQ$^{22}$) or its *N*-acetylated version attached C-terminally to the solid support, like in solution experiments, was not effectively recognised by MAb 996. Its elongated derivative on the C-terminus had no interaction with MAb 996 either.

The presence of Pro at position 18 resulted in a peptide which was highly capable to bind 996 monoclonal antibody. For the interpretation of these data several acetylated peptides were also studied. The comparison of binding data suggests that the blocking of free $NH_2$-group at the N-terminus by acetylation (Ac-$^{19}$TGTQ$^{22}$ vs. $^{19}$TGTQ$^{22}$ and Ac-$^{18}$PTGTQ$^{22}$ vs. $^{18}$PTGTQ$^{22}$) can slightly increase the peptide–996 antibody interaction. However, the presence of Pro at position 18 is essential ($^{18}$PTGTQ$^{22}$ vs. Ac-$^{19}$TGTQ$^{22}$) for pronounced antibody binding which cannot be achieved by its replacement with an acetyl group. These data also showed that the N-terminal elongation of the peptide does not affect the 996 recognition as much as in solution. There is no significant difference between the recognition of the C-terminally pin-bound Ac-$^{16}$PTPTGTQ$^{22}$ and $^{18}$PTGTQ$^{22}$ peptides.

This observation together with the findings of the RIA experiments shows that peptide $^{19}$TGTQ$^{22}$ representing the deduced antibody epitope is not recognised by MAb 996. For the antibody recognition of the $^{19}$TGTQ$^{22}$ motif an amide group is required on the N-terminus, either in the form of the

Pro at position 18 or as an acetyl group. For the optimal 996 recognition the $^{16}$PTP$^{18}$ sequence is required on the N-terminus of the $^{19}$TGTQ$^{22}$ epitope.

Considering the above data it can be concluded that the 996 monoclonal antibody recognises the X-$^{19}$TGTQ$^{22}$ peptide as a minimal epitope, where X = Ac or Pro from the native sequence.

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## REFERENCES

1. Y.S. Kim, J.R. Gum, J.C. Byrd and N.W. Toribara (1991). The structure of human intestinal apomucins. *Am. Rev. Respir. Dis. 144*, S10–S14.
2. M.R. Price, M. Sekowski, G.-Y. Yang, L.G. Durrant, R.A. Robins and R.W. Baldwin (1991). Reactivity of anti-(human gastric carcinoma) monoclonal antibody with core-related peptides of gastrointestinal mucins. *Cancer Immunol. Immunother. 33*, 80–84.
3. S. Gendler, J. Taylor-Papadimitriou, T. Duhig, J. Rothbard and J. Burchell (1988). A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. *J. Biol. Chem. 263*, 12820–12823.
4. J.R. Gum, J.C. Byrd, J.W. Hicks, N.W. Toribara, D.T.A. Lamport and Y.S. Kim (1989). Molecular cloning of human intestinal mucin cDNAs. *J. Biol. Chem. 264*, 6480–6487.
5. J.R. Gum, J.W. Hicks, D.M. Swallow, R.L. Lagace, J.C. Byrd, D.T.A. Lamport, B. Siddiki and Y.S. Kim (1990). Molecular cloning of cDNAs derived from a novel human intestinal mucin gene. *Biochem. Biophys. Res. Comm. 171*, 407–415.
6. N. Porchet, N. Van Cong, J. Dufosse, J.-P. Audie, V. Guyonnet Duperat, M.S. Gross, C. Denis, P. Degand, A. Bernheim and J.-P. Aubert (1991). Molecular cloning and chromosomal localization of a novel human tracheo-bronchial mucin cDNA containing tandemly repeated sequences of 48 base pair. *Biochem. Biophys. Res. Comm. 175*, 414–422.
7. D. Meerzeman, P. Charles, E. Daskal, M.H. Polymeropoulos, B.M. Martin and M.C. Rose (1994). Cloning and analysis of cDNA encoding a major airway glycoprotein, human tracheobronchial mucin (MUC5). *J. Biol. Chem. 269*, 12932–12939.
8. N.W. Toribara, A.M. Roberton, S.B. Ho, W.-L. Kuo, E. Gum, J.W. Hicks, J.R. Gum, Jr., J.C. Byrd, B. Siddiki and Y.S. Kim (1993). Human gastric mucin. *J. Biol. Chem. 268*, 5869–5885.
9. L.A. Bobek, H. Tsai, A.R. Biesbrock and M.J. Levine (1993). Molecular cloning, sequence, and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). *J. Biol. Chem. 268*, 20563–20569.
10. K. Uray, M.R. Price, L.G. Durrant, J. Kajtár and F. Hudecz in: *Peptides 1992*, C.H. Schneider and A.N. Eberle, Eds., p. 871–872, ESCOM, Leiden 1993.
11. L.G. Durrant, E. Jacobs and M.R. Price (1994). Production of monoclonal antibodies recognising the peptide core of MUC2 intestinal mucin. *Eur. J. Cancer 30A*, 355–363.
12. H.M. Geysen, R.H. Meloen and S.J. Barteling (1984). Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proc. Natl. Acad. Sci. USA 81*, 3998–4002.
13. M.R. Price, M. Sekowski, A. Ladányi, K. Uray, Y. Ma, L.G. Durrant and S.J.B. Tendler (1993). Immune recognition of human colonic-tumour-associated MUC-2 mucins using an anti-peptide antibody. *Int. J. Cancer 55*, 753–759.
14. S.-S. Wang (1973). *p*-Alkoxybenzyl alcohol resin and *p*-alkoxybenzyloxycarbonyl-hidrazide resin for solid phase synthesis of protected peptide fragments. *J. Am.Chem. Soc. 95*, 1328–1333.
15. B. Penke and J. Rivier (1987). Solid-phase synthesis of peptide amides on a polystyrene support using fluorenylmethoxycarbonyl protecting groups. *J. Org. Chem. 52*, 1197–1200.
16. E. Kaiser, R.L. Colescott, C.D. Bossinger and P.I. Cook (1970). Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem. 34*, 595–598.
17. V. Krchnák, J. Vágner, P. Safár and M. Lebl (1988). Noninvasive continuous monitoring of solid-phase peptide synthesis by acid–base indicator. *Collection Czechslovak Chem. Commun. 53*, 2542–2548.
18. J.C. Jensenius and A.F. Williams (1974). The binding of anti-immunoglobulin antibodies to rat thymocytes and thoracic duct lymphocytes. *Eur. J. Immunol. 4*, 91–97.

 