Preparation of MUC-1 Oligomers Using an Improved Convergent Solid-phase Peptide Synthesis*

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The sequentially repeating nature of the core mucin polypeptide chain MUC-1 on the surface of malignant cells makes it an excellent target for cancer immunotherapy. We describe a reliable and efficient method of synthesizing oligomers, up to five tandem repeats and oligomer heterotope derivatives with a 15-amino acid epitope from tetanus toxin using an improved convergent solid-phase peptide synthesis. The different oligomers were easily distinguishable by reverse-phase high pressure liquid chromatography, but they were poorly fixed and migrated with the same migration rate, irrespective of size, in electrophoretic studies. In contrast, the oligomer heterotopes exhibited size-dependent electrophoretic behavior but in high pressure liquid chromatography chromatograms the different heterotopes were eluted simultaneously in two peaks representing the α- and β-enantiomers of the derivatives. The oligomer heterotopes were recognized as antigens in Western blotting with a murine monoclonal antibody against the epitope APDTR. In enzyme immunoassay studies with the same antibody an increasing reactivity was observed against the larger oligomers and confirmed by inhibition assays as the MUC-1 pentamer was the most efficient inhibitor. These results support the suggestion that the pentamer attains a structure closer to the native conformation and is more immunogenic. In conclusion, large composite peptides can be reliably synthesized with the convergent solid-phase peptide strategy offering an attractive option to vaccine design and development.

The implication of the mucin MUC-1 in many human tumors of epithelial origin has generated great interest in recent years, particularly as a target for immunotherapy (1–3). The core protein of MUC-1 is composed of a number of tandem repeats, each consisting of a 20-amino acid (aa)1 peptide (4, 5). Murine monoclonal antibodies raised against MUC-1 appear to react selectively with breast cancer mucins but not with normal mucin (6, 7) and are targeted principally to the epitope APDTRP of the 20-mer peptide (8–10).

The exposure of the MUC-1 tandem repeats on the cell surface of malignant cells combined with the ability of the immune system to respond specifically to these peptide epitopes offers an ideal opportunity to rationally design appropriate synthetic vaccines to target this tumor-associated antigen. Recent vaccination studies in humans using a MUC-1 dimer conjugated with diphtheria toxoid showed that the formulation used was not sufficiently immunogenic (11). This, perhaps, is not surprising as one or two repeats do not attain a native conformation, whereas peptides consisting of three or more repeats take a rod-shaped ordered structure (12). A five-repeat peptide has been reported to induce enhanced antigenicity (13), and the results of a phase I vaccination trial were encouraging (14). In the present study we exploited the recent advances in convergent solid-phase synthesis (CSPP) to reliably prepare and evaluate MUC-1 oligomers up to five repeats and MUC-1 oligomer derivatives with a 15-aa epitope from tetanus toxin.

EXPERIMENTAL PROCEDURES

Materials—The 20-mer MUC-1 fragment 6 and the 15-mer ttp fragment 7 (Fig. 1) as well as smaller MUC-1 fragments (Table I) were synthesized by an automated solid-phase Fmoc strategy (430A Peptide Synthesizer, Applied Biosystems) with the N-methylpyrrolidinone-di-(ethylene)carboxyldiimide (NMP-DCC) method according to the manufacturer’s instructions, modified to take 2-chlorotriyl chloride resin (CLTR). The solid-phase support was in all cases CLTR. Protected amino acids and CLTR were obtained from CBL Patras (Patra, Greece). NMP, dimethylformamide (DMF), dichloromethane (DCM), acetonitrile, methanol, trifluoroacetic acid, acetic acid, and diisopropylcarbodiimide (DIC) were obtained from Merck (Germany). DCC, ethanedithiol, thioanisole, phenol, piperidine, trifluoroethanol (TFE), and diisopropylthalamine (DIPEA) were obtained from Sigma.

Synthesis of Protected Fragments 6 and 7—For the synthesis of the required protected fragment 6 and of the protected fragment 7 (Fig. 1) we used the labile Fmoc-7-butyl amino acids. Trypt was applied for the protection of the side chains of His and Ser. Pmc (2,2,5,7,8-penta-methylchroman-6-sulfuryl) was applied for Arg. This protection scheme allowed the mildest possible deprotection conditions of the final protected peptide. The solid support CLTR was chosen because it effectively suppresses diketopiperazin formation during the synthesis of peptides containing Pro (15–17) at the C terminus, and in addition the protected peptides synthesized on this resin could be cleaved quantitatively under extreme mild conditions in the absence of acids (18).

The synthesis of 6 was initiated with the esterification of Fmoc-Pro-OH 1 on 1 g CLTR 2 with DIPEA in DMF, to yield Fmoc-Pro-O-CLTR 3. Unreacted remaining trityl chloride functions were converted to the corresponding methyl ether by washing the resin three times with methanol/DIPEA/DCM (15:5:80) for 5 min each. Fmoc removal was performed immediately after the end of the esterification procedure by treating with 25% piperidine in DCM to obtain resin 4 with a substitution of 0.3 mmol of Fmoc of resin. The peptide chain was elongated using DCC/HOBt as the condensing agent, NMP as the solvent, and 25% piperidine in NMP for the removal of Fmoc. Finally, the obtained resin peptide ester 5 (Fig. 1) was treated at room temperature for 60 min with a 3:7 mixture of TFE/DCM. Protected peptide 6

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was recovered after solvent evaporation in vacuum, precipitation with water, filtration and drying in ether. The protected ttp 7 was synthesized by similar methods.

Synthesis of MUC-1 Oligomers and ttp-MUC-1 Heterotope Derivatives by CSPP—A small scale pilot synthesis was performed by condensing a 2-fold molar excess of protected fragment 6 over the resin-bound amino component 5. The protected fragment was applied as a 0.05 M solution in Me$_2$SO and precipitated in vacuum, followed by filtration and drying in ether. The protected ttp 7 was synthesized by similar methods.

In a second CSPP preparation of the 5-mer MUC-1 we started with a new synthesis of resin 5 that condensed with the protected MUC-1 6 four times in succession, introducing the following modifications: (i) a lower proline substitution in resin 5 (0.1 mmol/g resin); (ii) a more concentrated solution of 6 (0.1 M); (iii) each fragment was applied in three-fold molar excess; and (iv) DCC was replaced by DIC as the dehydrating agent.

Cleavage, Deprotection, and Isolation of Peptides—After the final coupling step, all synthesized peptides were treated with 25% piperidine for removal of the Fmoc, washed with diethyl ether, and dried. The cleavage and deprotection was performed with a trifluoroacetic acid: water:phenol:ethanedithiol:thioanisole solution (82.5:5.5:2.5:2.5) for 2 h at room temperature. The crude peptides were precipitated in ice-cold ether, washed several times with ether, dried, dissolved in water, lyophilized, and stored at −20 °C until used. The peptides synthesized for the present study are listed in Table 1.

HPLC Analysis, SDS-PAGE Electrophoresis and Western Blotting—HPLC was performed in the Waters 600 LC system (Waters) with a UV 486 detector using a semipreparative C18 column (Synchropack RP 250 × 7.8-mm, Synchro Inc.).

SDS-PAGE electrophoresis was performed with the Phast system (Amersham Pharmacia Biotech, Sweden), and the gels were stained with silver nitrate according to the manufacturer’s instructions.

Gel transfer to nitrocellulose (Biotrace NT, Gelman Sciences, Germany) was performed with passive diffusion with transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol) for 15 min. The membranes were either silver-stained directly (20) or blocked with 2% gelatin in 50 mM PBS, pH 7.0, for 1 h.

FIG. 1. Strategy for Fmoc synthesis of basic peptide fragments. Fmoc, N-(9-fluorenyl)methoxycarbonyl; DIPEA, diisopropylethylamine; DCM, dichloromethane; CLTR, 2-chlorotrityl chloride resin; TFA, triethylamine; Gly, glycine; Val, valine; Thr, threonine; Ser, serine; Lys, lysine; Leu, leucine; Phe, phenylalanine; His, histidine; Arg, arginine; Asp, aspartic acid; Pro, proline; Gly, glycine; Ser, serine; Trt, trityl; Boc, tert-butyloxycarbonyl.

FIG. 2. Fragment condensation strategy for the synthesis of MUC-1 oligomers and ttp-MUC-1 heterotope derivatives.
at room temperature and washed with 0.1% Tween in PBS. Murine monoclonal anti-MUC-1 antibody (BC-2) in PBS with 0.1% bovine serum albumin were added to each well (100 µl) and incubated for 1 h at room temperature. The BC-2 antibody (IgG1) with specificity to peptide epitope APDTR of MUC-1 (1) was a gift from Dr. P.-X. Xing (Austin Research Institute, Heidelberg, Australia). After washing off excess antibody, a second incubation with a rabbit anti-mouse antibody-peroxidase conjugate (100 µl) followed for 1 h at room temperature. After four washes with PBS the antigen-antibody reaction was revealed by adding to each well 100 µl of substrate (0.25 mM tetramethyl benzidine, 0.03% v/v hydrogen peroxide in 50 mM sodium acetate buffer, pH 5.2) (23). The reaction was stopped after 15 min by the addition of 50 µl 2 M sulfuric acid per well. The absorbance was measured at 450 nm (Multiscan, Flow Labs, Finland).

Inhibition Enzyme Immunoassay—The monoclonal anti-MUC-1 antibody BC-2 was diluted 1:6000 in PBS with 0.1% bovine serum albumin were added to each well (100 µl) and incubated for 1 h at room temperature. The BC-2 antibody (IgG1) with specificity to peptide epitope APDTR of MUC-1 (1) was a gift from Dr. P.-X. Xing (Austin Research Institute, Heidelberg, Australia). After washing off excess antibody, a second incubation with a rabbit anti-mouse antibody-peroxidase conjugate (100 µl) followed for 1 h at room temperature. After four washes with PBS the antigen-antibody reaction was revealed by adding to each well 100 µl of substrate (0.25 mM tetramethyl benzidine, 0.03% v/v hydrogen peroxide in 50 mM sodium acetate buffer, pH 5.2) (23). The reaction was stopped after 15 min by the addition of 50 µl 2 M sulfuric acid per well. The absorbance was measured at 450 nm (Multiscan, Flow Labs, Finland).

RESULTS AND DISCUSSION

The repetition of the MUC-1 20-mer peptide is an attractive model with which to study the efficiency of the convergent approach for the solid-phase synthesis of large peptides and small proteins (24). The method is based on the principle that protected peptide fragments corresponding to the entire protein sequence can be condensed sequentially on a suitable solid support. We applied this method for the synthesis of MUC-1 oligomers using Fmoc amino acids and 2-chlorotrityl resin as the solid support (15).

Our synthesis strategy for the preparation of MUC-1 oligomers utilized the sequential condensation of the protected peptide 6 (Figs. 1 and 2). The aa sequence of this peptide was designed to contain the immunodominant epitope APDTR in the interior of the peptide so that each repeated unit would contain one epitope and prolines at positions 1 and 20. The rationale for our choice was that: (i) the optical stability of proline at the C-terminal position of the electrophilically activated fragment would ensure the optical purity of the resulting oligomers; (ii) fragment 6 was soluble in the solvent used (Me3SO) as required for a successful solid-phase condensation reaction; and (iii) both the free NZ-function of the resin-bound amino component as well as the C-terminal function of the applied carboxy component were exposed and easily reached by the other reactants. This could not be the case if completely formed β-turns were contained in one of the reactive terminal positions. We anticipated that the presence of several β-turn...
Chain elongation starting from the resin the extremely acid-sensitive bond of the amino acid to the resin hydrogen chloride, which subsequently affects the cleavage of trityl chloride residues. This in turn could cause evolution of amino acid from the resin due to slow hydrolysis of unreacted amino function soon after the esterification reaction by treatment with 25% piperidine in DMF. This step was considered especially at both N- and C-terminal positions of the 20-mer, for the guanidino function of Arg (25). Completion of the coupling reactions was determined by the Kaiser test and of the Fmoc removal by TLC. Due to the presence of the very sensitive and β-sheet destructive Pro residues in the peptide chain, especially at both N- and C-terminal positions of the 20-mer, would impede β-turn formations and enhance peptide solubility. The selected MUC-1 20-aa protected sequence had the required reactivity at both terminal positions.

The protected 20-aa fragment was synthesized starting with the esterification of Fmoc-Pro-OH with CLTR (Fig. 1) (15). The obtained resin-bound proline derivative, with a loading of 0.3 mmol of Pro/g of resin. This first preparation showed incomplete condensations during the synthesis of the di-, tri-, tetra- and pentamers 9, 11, 13, 15 (Fig. 3, b-e). HPLC analysis of the final deprotected crude products indicated that condensation of 6 without prior reprecipitation from TFE/water (26). Similarly, the protected ttp 7 was synthesized in 95% yield and 96% purity.

Initially, we commenced the synthesis of MUC-1 oligomers using the resin 5 with a loading of 0.3 mmol of Pro/g of resin. We used a lower proline substitution (0.1 mmol/g resin) because in proceeding with the condensation reactions, the peptide/resin weight ratio increased and considerably altered the swelling properties of the resin. Resins with a high peptide loading reveal an increased polarity of the polystyrene matrix. Therefore, movement of large polar molecules such as the free protected peptide fragments, is restricted through polar interactions with support and can reach only with difficulty the reactive NH₂-terminal positions of the resin-bound amino component. We also applied a more concentrated solution of 6 and three-fold molar excess of fragment 6 over the resin-bound component 5, using the condensing agent DIC/HOBt (1:1.5) in Me₂SO instead of DCC. This aspect was considered important as we speculated that during the long-lasting condensation reactions dicyclohexyl urea could precipitate and block the resin pores hindering the movement of the protected fragments inside the resin. The improved condensation yields that resulted from the above changes allowed us to prepare the peptide oligomers on a larger scale. The starting resin 5 (0.1 mmol/g resin) was condensed with the protected MUC-1 6 four times in succession thus resulting in the formation of the pentamer MUC-1. The yield and purity of the crude MUC-1 pen-
tamer peptide were satisfactory as shown by HPLC analysis (Fig. 4b) and compared with the first preparation (Fig. 4c) thus allowing us to obtain the pentamer in a highly purified form after HPLC purification (Fig. 4a). The molecular weight of the MUC-1 pentamer determined by mass spectroscopy (Fig. 5) was in agreement with the expected molecular weight as calculated from the amino acid content.

Electrophoretic Analysis of the MUC-1 and ttp-MUC-1 Oligomers—The synthetic MUC-1 oligomer peptides exhibited unusual behavior in SDS-PAGE. The peptides could not be visualized directly on the polyacrylamide gels because they could not be fixed by methanol/acetic acid, glutaraldehyde, or trichloroacetic acid. The electrophoretic properties were studied when the oligomers were transferred to a nitrocellulose membrane with passive diffusion immediately after the electrophoretic separation and by the silver staining, which followed (20). The monomer and dimer peptides were not visualized at all, probably due to insufficient amounts being transferred onto the nitrocellulose (Fig. 6, lanes 1 and 2). The other oligomers (3-mer, 4-mer, and 5-mer) became clearly visible. They appeared to migrate slowly with the same electrophoretic rate (Fig. 6, lanes 3–5) although the oligomers differed from each other by 20 and 40 aa in length. This anomaly may be attributed to the high proline content and alanine-proline sequences that have

![Fig. 8. HPLC analysis of ttp-(MUC-1)1-3 showing increasing racemization as the length of MUC-1 oligomers increased; (a), ttp-(MUC-1)3; (b), ttp-(MUC-1)2; (c), ttp-(MUC-1)1. HPLC was performed in a semipreparative C18 column, and the eluent was monitored at 220 nm. An elution gradient from acetonitrile containing 0.1% trifluoroacetic acid:water was applied for 30 min at a flow rate of 1 ml/min, from 15 to 40% acetonitrile.](image)

![Fig. 9. Enzyme immunoassay reactivity of MUC-1 oligomers against a monoclonal antibody (BC-2), which recognizes the APDTRP epitope.](image)

![Fig. 10. Competitive inhibition enzyme immunoassay of the APDTRP-recognizing monoclonal antibody by a range of synthetic peptides. The plates were coated with the MUC-1 pentamer.](image)

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### Table I

| Peptides synthesized and used in the study |
|-------------------------------------------|
| MUC-1 (20 aa)                              |
| MUC-1 immunodominant epitope (ID) (6 aa)    |
| 9-aa fragment containing the ID epitope    |
| 9-aa fragment without the ID epitope       |
| Tetanus toxin peptide (ttp) 15 aa          |
| MUC-1 oligomers synthesized by fragment condensation |
| MUC-1 1-mer 20 aa                          |
| MUC-1 2-mer 40 aa                          |
| MUC-1 3-mer 60 aa                          |
| MUC-1 4-mer 80 aa                          |
| MUC-1 5-mer 100 aa                         |
| ttp-MUC-1 heterotope derivatives synthesized by fragment condensation |
| ttp-(MUC-1)1, 35 aa                        |
| ttp-(MUC-1)2, 55 aa                        |
| ttp-(MUC-1)3, 75 aa                        |
| ttp-(MUC-1)4, 95 aa                        |
| ttp-(MUC-1)5, 115 aa                       |
| PAHGVTSAUDPTRPGAAGSTAP                     |
| APDTRP                                     |
| SAPDTRP                                    |
| STAPPANHGV                                 |
| QYIKANSFIGITEL                             |
| (PAHGVTSAUDPTRPGAAGSTAP)1                  |
| (PAHGVTSAUDPTRPGAAGSTAP)2                  |
| (PAHGVTSAUDPTRPGAAGSTAP)3                  |
| (PAHGVTSAUDPTRPGAAGSTAP)4                  |
| (PAHGVTSAUDPTRPGAAGSTAP)5                  |
| QYIKANSFIGITEL−(PAHGVTSAUDPTRPGAAGSTAP)1   |
| QYIKANSFIGITEL−(PAHGVTSAUDPTRPGAAGSTAP)2   |
| QYIKANSFIGITEL−(PAHGVTSAUDPTRPGAAGSTAP)3   |
| QYIKANSFIGITEL−(PAHGVTSAUDPTRPGAAGSTAP)4   |
| QYIKANSFIGITEL−(PAHGVTSAUDPTRPGAAGSTAP)5   |
been reported to alter the charge and conformation of the SDS-polypeptide complex (27–29).

In contrast, the ttp-MUC-1 heteroantibody derivatives showed normal electrophoretic behavior, probably due to the influencing presence of the ttp fragment (Fig. 7). These peptides were easily fixed onto polyacrylamide gels and stained with silver nitrate. Electrophoretic studies from the first preparation of the ttp-MUC-1 heteroantibody derivatives (Table I) showed the anticipated ascending molecular weight order from ttp to ttp-5-mer (Fig. 7, lanes 1–8) and clearly demonstrated the incompletion of the reactions during the synthesis of the 3-mer, 4-mer, and 5-mer as had also been observed by HPLC analysis. It was noted that the ttp-MUC-1 heteroantibody derivatives could not be separated by HPLC as they were all eluted simultaneously from the gradient systems we applied (Fig. 8). This phenomenon may be attributed to the dominating hydrophobic nature of the ttp peptide, equally affecting the elution of the different derivatives.

As expected, the condensation of the protected ttp fragment with the resin-bound MUC-1 oligomers proceeded with the partial racemization of the C-terminal leucine residue.

In fact, we observed that two distinct peaks were always present in the reverse phase-HPLC elution profile for each derivative. The two components corresponding to the L- and D-enantiomers of the derivatives were separated by semi-preparative HPLC. Both showed identical electrophoretic properties (Fig. 7, lanes 6 and 7), exactly the same molecular weight and the same amino acid ratio as determined by aa analysis of the ttp-MUC-1 5-mer. The percentage of racemization appeared to increase in proportion to the length of MUC-1 oligomer. This was due to the slower fragment condensation reactions leading to a longer exposure of the activated ttp in the condensation prone benzotriazoyl ester state prior to condensation (Fig. 8).

Immunological Properties of MUC-1 Oligomers—The murine monoclonal antibody BC-2 was titrated against equal amounts of MUC-1 oligomers in enzyme-linked immunosorbent assay (Fig. 9). The results showed a characteristic preference of the antibody for the oligomers and much less for the monomer in agreement with previous observations (12). This was also demonstrated in Western blotting using a mixture of the ttp-MUC-1 oligomers (Fig. 7, lane 9) where ttp-(MUC-1)_{5} did not give any visible immunoreaction, whereas all the other oligomer derivatives reacted with the monoclonal antibody. Finally, in an inhibition enzyme immunoassay study of the monoclonal antibody by a range of peptides (Fig. 10 and Table I), it was demonstrated that free MUC-1 oligomers were more efficient inhibitors than the monomer and the APDTRP epitope. The control peptide STAPPAHGV, which represented a region of MUC-1 exclusive of the immunodominant epitope, did not react with the antibody. These results strongly support the suggestion that as the number of MUC-1 tandem repeats increases, the epitopes may attain a structure closer to native conformation of unglycosylated mucin and become more immunogenic (12).

Synthesis of large peptides such as the ones required for immunotherapeutic and vaccination studies is difficult to achieve with a sequential extension approach as the reaction efficiency decreases dramatically with the increasing size of the peptide beyond 30–40 aa, affecting both purity and yields. Recent advances in peptide chemistry for cleaving peptides from resins under extremely mild conditions allowed us to adopt an improved convergent solid-phase peptide synthesis strategy and prepare MUC-1 oligomers under controlled conditions up to five repeats and derivatives of the same oligomers with a tetanus toxin T cell epitope. HPLC and mass spectrometry analysis confirmed the purity and correct size of the pentamer MUC-1, and immunological studies demonstrated that the oligomers were recognized as specific antigens by anti-mucin antibodies. The construction of large composite synthetic peptides, such as those demonstrated here, offers a rational approach to designing and studying synthetic vaccines with defined structural and accessory epitopes as a means of evoking precise immune responses, which are required in immunotherapy.

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