

Identification of the Human Melanoma-associated Chondroitin Sulfate Proteoglycan Antigen Epitope Recognized by the Antitumor Monoclonal Antibody 763.74 from a Peptide Phage Library

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ABSTRACT

To identify the epitope of the melanoma-associated chondroitin sulfate proteoglycan (MCSP) recognized by the monoclonal antibody (mAb) 763.74, we first expressed random DNA fragments obtained from the complete coding sequence of the MCSP core glycoproteins in phages and selected without success for binders to the murine mAb 763.74. We then used a library of random heptapeptides displayed at the surface of the filamentous M13 phage as fusion protein to the NH₂-terminal portion of the minor coat protein III. After three rounds of selection on the bound mAb, several phages displaying related binding peptides were identified, yielding the consensus sequence Val-His-Leu-Asn-Tyr-Glu-His. Competitive ELISA experiments showed that this peptide can be specifically prevented from binding to mAb 763.74 by an anti-idiotypic MK2–23 mouse:human chimeric mAb and by A375 melanoma cells expressing the antigen MCSP. We screened the amino acid sequence of the MCSP molecule for a region of homology to the consensus sequence and found that the amino acid sequence Val-His-Ile-Asn-Ala-His spanning positions 289 and 294 has high homology. Synthetic linear peptides corresponding to the consensus sequence as well as to the MCSP-derived epitope inhibit the binding of mAb 763.74 to the phages displaying the consensus amino acid sequence. Finally, the biotinylated consensus peptide adsorbed to streptavidin-microtiter plates can be used for the detection of mAb 763.74 in human serum. These results show clearly that the MCSP epitope defined by mAb 763.74 has been identified.

INTRODUCTION

The high-molecular weight melanoma-associated antigen (1), also identified as MCSP² (2, 3), is a major marker for melanoma (4) and is highly expressed at the surface of the tumor cells (1, 5). The high immunogenicity of the MCSP molecule in mice has allowed the preparation of several mAbs (5–10). One of those, mAb 763.74, has been used to generate an anti-id mAb, MK2–23 (9). After conjugation to a protein carrier and administration with an adjuvant, this anti-id mAb induced a specific humoral response in patients with advanced melanoma (1, 11). The treatment significantly increased the survival of these patients (1, 11). The clinical results obtained render the identification of the antigenic structure of the proteoglycan MCSP as mimicked by MK2–23 and recognized by mAb 763.74 of great interest, especially because the antigen, despite being cloned, has not been expressed as yet (12). The epitope or a peptide mimetic for the former could advantageously replace the antigen in an ELISA to be performed on clinical samples obtained from patients immunized with the mAb MK2–23 conjugate.

In the last 5 years, the phage display technology has provided a new approach to exploring epitopes recognized by monoclonal or poly-

clonal antibodies (13–18). Libraries of random peptides can be expressed either as linear or as constrained structures at the NH₂ terminus to the M13 minor coat protein III or major coat protein VIII and displayed at the surface of infectious bacteriophage M13 (19, 20). With the same technology, fragments generated after random DNase I digestion of a cDNA can be cloned and expressed as a fusion protein to one of the coat proteins of the bacteriophage (21–23). Thus, in the present study, we expressed random DNA fragments obtained from the complete coding sequence of the MCSP glycoprotein in phages and selected for binders to the murine mAb 763.74. In a parallel approach, we also selected linear peptides displayed by the minor coat protein III from a random heptapeptide phage library. For both libraries, we used the selection procedure we had developed and optimized earlier (24).

MATERIALS AND METHODS

Cell Lines, Bacterial Strains, MCSP Protein Fragments, and Heptapeptide Display Phage Libraries. The melanoma cell line A375metS3,4, which expresses the MCSP antigen, is a derivative of the parental cell line A375-Met-Mix (25), and are hereafter designated A375. The S7 cells are subclones of the cell line SK-Mel-25 (26), and they do not express the antigen. The cells used in our experiments were obtained from Dr. J. Mestan and Dr. J. Brügggen (Novartis Pharma AG, Basel, Switzerland).

Plasmids encoding seven overlapping fragments covering the entire cDNA of the MCSP core glycoprotein were obtained as described (12) and kindly provided by M. Vanek (Novartis Pharma AG, Basel, Switzerland).

The phagemid pTK3552B used for displaying the MCSP-derived protein fragments at the surface of the bacteriophage M13 has been described earlier (27). The construction of the library is described below. The VSCM13 helper phage (Stratagene, La Jolla, CA) was used for phagemid rescuing as also described (27).

The library displaying the random linear heptapeptide at the surface of the M13 phage as fusion with the minor coat protein III was part of the Ph.D. kit system of New England Biolabs (Beverly, MA). The *Escherichia coli* host strain XL1-Blue (Stratagene) was used throughout the experiments presented in this study.

Construction of a Phage Library Displaying Fragments of the MCSP Core Glycoprotein after Random DNase I Digestion of the Encoding cDNAs. Each of the seven plasmids encoding the cDNA for the MCSP was partially digested with DNase I in the presence of Mn²⁺ as described in the NovaTope digestion protocol (Novagen, Madison, WI). After the digestion, the DNA was separated on a 2% agarose gel. DNA fragments between 100 and 700 bp were isolated and purified from a preparative agarose gel with the help of the QIAex II kit (Qiagen, Chatsworth, CA). The vector pTK3552B and the different fragments were digested to completion with *Xho*I and *Spe*I, and both the DNA of the vector and the DNase I-generated fragments were treated with 2 U of vent DNA polymerase (New England Biolabs) in 100 μ l of the supplied buffer in the presence of 0.2 mM of the four deoxynucleotides for 20 min at 72°C. After several blunt end ligations, all done with the Boehringer rapid ligation kit, the DNA was electroporated into *E. coli* XL1-Blue cells. If the total number of transformants after titration was higher than 2×10^4 independent clones, the cells were inoculated into 200 ml of Luria Broth containing 50 μ g/ml ampicillin and 10 μ g/ml tetracycline. After overnight growth of the cells, phages were rescued with the helper phage VCSM13 and precipitated twice with 16.7% PEG 8000 and 3.3 M NaCl as described (28).

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² The abbreviations used are: MCSP, melanoma-associated chondroitin sulfate proteoglycan; mAb, monoclonal antibody; anti-id, anti-idiotypic; PEG, polyethylene glycol; TBS, Tris-buffered saline; aa, amino acid.

Biopanning of the Phage Libraries Displaying either the MCSP Protein Fragments or the Random Linear Heptapeptides. Coating, blocking, washing, and elution of the phages using 5-ml Maxisorb immunotubes (Nunc, Basel, Switzerland) was performed as published previously (24). The MCSP protein fragment phage library ($1-4 \times 10^{10}$ phages) was first panned against mAb 763.74 coated at a concentration of $50 \mu\text{g/ml}$. The coating concentration of the antibody was reduced to $1 \mu\text{g/ml}$ for the second round and to 100 ng/ml for the third round. Finally, a fourth round with a coating of 10 ng/ml mAb 763.74 was conducted. After elution, the phages were amplified, precipitated twice with PEG 8000/NaCl, and titered on *E. coli* XL1-Blue cells.

The linear heptapeptide library ($10 \mu\text{l}$; 2×10^{11} phages) from the Ph.D. kit (Bioconcept Allschwil, Switzerland), with a diversity of 2×10^9 individual peptides, was added to tubes prepared as described above for the selection of phages containing MCSP fragments but with a coating concentration of $100 \mu\text{g/ml}$, $1 \mu\text{g/ml}$, and 2 ng/ml , respectively, for the three rounds of selection. The recovered phages were added to exponentially growing *E. coli* XL1-Blue cells at $A_{600 \text{ nm}} = 0.6-0.8$. After the infection, the cells were grown at 37°C for 20 h, and the phages present in the growth medium were purified with PEG 8000/NaCl precipitation. The amplified phages were titered in *E. coli* XL1-Blue cells (28). From individual clones, we prepared phages from the supernatant of the growth medium. The phages were precipitated and concentrated with PEG 8000/NaCl.

Chemiluminescence ELISA. The wells of a Microtiter 2-immunoassay plate (Dynatech Laboratories, Chantilly, VA) were coated with $2 \mu\text{g/well}$ of mAb 763.74, BSA, or with irrelevant IgG1 antibodies serving as negative controls in 50 mM Tris-HCl (pH 8.1). After the wells were blocked with Blotto (5% nonfat dry milk and 0.05% Tween 20 in TBS), the phages were added for 2 h at 37°C . The wells were washed 10 times with TBS containing 0.05% Tween 20, and anti-M13 antibody conjugated to horseradish peroxidase (Pharmacia, Uppsala, Sweden) was added at a 1:4000 dilution in Blotto. After incubation for 1 h at room temperature and the same washing steps as described above, luminol, a chemiluminescent substrate (BM Chemiluminescence ELISA Reagent, Boehringer Mannheim, Mannheim, Germany) was added. Readings of emitted light were recorded as relative units (Microlumet LB96P Berthold, Regensdorf, Switzerland).

In competitive assays with phages displaying mAb 763.74-binding peptides, the phages were incubated either with a decreasing concentration of the competitor anti-id mAb MK2-CH γ 1 or one of two different irrelevant antibodies for 1 h at room temperature prior to their addition to wells coated with $0.5 \mu\text{g/well}$ of mAb 763.74. The competitor mAb MK2-CH γ 1 referred to here is a chimeric mouse:human antibody derivative of the original MK2-23 (29). The binding of phages in the presence of the competitor antibody was monitored with an anti-M13 antibody conjugated to horseradish peroxidase as described above. In other competitive ELISA experiments, we first coated the wells of the microtiter plate with 1×10^{11} phages. The preincubation mix, composed of the mAb 763.74 ($10 \mu\text{g/ml}$) and either the different competitor peptides or the competitor anti-id mAb MK2-CH γ 1, was added to the coated and blocked wells. In some experiments, we coated the wells with $0.5 \mu\text{g}$ of streptavidin. After the wells were blocked with Blotto and washed, $0.016 \mu\text{g/well}$ of the VHLNYEH-SGSGK(biotin) peptide in Blotto was added for 1 h at room temperature. The binding of mAb 763.74 to the peptide was monitored by ELISA with an antimouse antibody conjugated to horseradish peroxidase.

In other experiments, the mAb 763.74 was coated at $0.5 \mu\text{g/well}$ and preincubated with different varying concentrations of competitor melanoma A375 cells expressing the MCSP protein or with MCSP-negative S7 cells for 1 h at 37°C . After the washing step described above, $5 \mu\text{M}$ of the VHLNYEH-SGSGK(biotin) peptide in Blotto was added to the wells. We measured the binding of the peptide to mAb 763.74 with a 1:100 dilution of a stock solution (1 mg/ml) of streptavidin conjugated to horseradish peroxidase in Blotto by ELISA.

DNA Sequence Analysis. After the third round of selection, the recovered peptide phages were titered on *E. coli* XL1-Blue cells. Thirty independent binding clones were analyzed by chemiluminescence phage ELISA, and 18 individual phages were chosen for DNA sequence analysis. After centrifugation, the replicative form of the phage DNA was isolated from the cells with the Qiagen maxi DNA kit. The DNA templates were used for automatic dideoxy-DNA sequencing with a LI-COR sequencing machine Model 4000 (MWG-Biotech, Ebersberg, Germany) and the Labstation Thermo Sequenase labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, Zurich,

Switzerland). The oligonucleotide primer 5'-CCC TCA TAG TTA GCG TAA CG-3' with the suitable fluorescence dye was found at position 96 downstream of the region encoding the random peptides in the library and was obtained from MWG-Biotech.

Peptide Synthesis. Peptides were synthesized on a Milligen 9050 Plus automated peptide synthesizer (continuous flow), using chemical protocols based on the fluorenylmethoxycarbonyl strategy. In all cases, the crude peptides were purified by reversed-phase medium-pressure liquid chromatography on a C_{18} column ($46 \times 3.6 \text{ cm}$, $15-25 \text{ mm}$ bead size; Lichroprep C-18) eluted with an acetonitrile-water gradient containing 0.1% trifluoroacetic acid. The purity of the final compounds was verified by reversed-phase analytical high-performance liquid chromatography, and the identity was assessed by correct mass spectral (matrix-assisted laser-desorption ionization time-of-flight mass spectrometry) and aa analyses.

Solubilization of MCSP from A375 Melanoma Cells and Treatment of A375 Cells with Endoglycosidases. A375 cells and MCSP-negative S7 cells were lysed in TBS (pH 8), containing 1% NP40 and a cocktail of protease inhibitors (Complete; Boehringer Mannheim). After centrifugation to remove insoluble material, aliquots ($100 \mu\text{g}$ of protein) of the lysate were treated with the enzymes *N*-glycosidase F (E.C. 3.5.1.52; Boehringer Mannheim) and chondroitinase ABC (E.C. 4.2.2.4; Sigma Chemical, St. Louis, MO), according to the manufacturers' instructions.

After termination of the enzyme treatment by boiling in the presence of 2% SDS and 5% 2-mercaptoethanol, samples were analyzed by SDS-PAGE and Western blotting.

RESULTS

Affinity Selection of a Phage Library Displaying Random MCSP Protein Fragments Using mAb 763.74. To identify the epitope recognized both by the primary mAb 763.74 and by its surrogate present in the anti-anti-id antibody response in immunized patients, we expressed random DNase I fragments derived from the different cDNA clones encoding the MCSP core glycoprotein at the surface of the bacteriophage M13.

At least 2×10^4 ($1.1 \times 10^3 \times 18$) independent transformants are necessary to create a library of expressed fragments large enough to isolate the epitope with a probability of 99.9% (28). This number is required because only 1 of 18 clones will be present in the library with a correct fusion between both the signal peptide and the minor coat protein III of the phages on both ends of the cloned DNase fragments, which themselves can be found in only one correct orientation ($3 \times 3 \times 2 = 18$). The library was converted to phages by rescuing with the helper phage VSCM13. After amplification, 1×10^6 copies of each individual fragment derived from the cloned MCSP core glycoprotein are present in the library. After the four rounds of selection, the amplified phage population was analyzed for binding to mAb 763.74 by chemiluminescence phage ELISA. There was no detectable binding signal, even with very high phage concentrations, implying that no phage was selected from the library (data not shown).

Earlier data suggested that the epitope should be a linear peptide (1, 30). According to the size distribution of the fragments expressed and displayed by the phage library, we would have expected to identify the linear peptide corresponding to the epitope after our selection procedure. We thus rechecked the possibility that the epitope or part of it is located on the oligosaccharide moiety of the MCSP molecule.

Solubilization of MCSP Molecule from Melanoma Cells and Endoglycosidase Treatment. After detergent lysis of the A375 cells, virtually all of the MCSP had been solubilized as indicated from Western blotting and staining with mAb 763.74. The antibody revealed a strong band at $M_r \sim 250,000$. The material from the MCSP-negative S7 cells did not show, as expected, reactivity with the antibody (data not shown).

The lysates were treated with *N*-glycosidase F to remove the N-Asn-linked oligosaccharides from the protein. Staining of the blots

revealed that after the digestion, the mAb 763.74-reactive MCSP band shifted to a lower apparent molecular weight, suggesting the removal of one or more N-linked oligosaccharides. The treatment of the lysate with chondroitinase ABC showed no effect on mobility or immunoreactivity of the MCSP band consistent with the idea that this band represents the core protein (data not shown and Ref. 1). These results demonstrate and confirm earlier results that the epitope recognized by mAb 763.74 is associated with the core protein but is not found on N-linked oligosaccharides.

Selection of a Linear Random Peptide Phage Library on mAb 763.74. Prompted by the negative results obtained with the MCSP protein fragments expressed and displayed by phages, we decided to pan a heptapeptide phage display library against mAb 763.74 in the search for a peptide mimetic of the epitope. After two rounds of selection, phages displaying peptide specifically binding to mAb 763.74 were enriched (Fig. 1). The phage population bound neither to BSA nor to other negative control antibodies. However, as observed previously for the gene fragment phage library, the heptapeptide phage bound to the negative control mAb ACA125. ACA125 is an anti-id mAb mimicking an epitope of the tumor-associated antigen CA-125 (31). In additional control experiments (data not shown), we could show that mAb ACA125 binds to the VSCM13 helper phage as well as to the pBCsk(+) phage (Stratagene). A panning round performed in the presence of an excess of soluble ACA125 did not change the binding characteristics of the phages to ACA125 bound to the microtiter plate (data not shown). Hence, the antibody is binding nonspecifically to the phage particles and not to the displayed peptides. This nonspecific binding was, however, useful as an internal experimental marker for the evaluation of the binding affinity of the selected phages. As expected, the third round of panning performed at a higher stringency of selection allowed the identification of phages binding more strongly to the antibody offered in the ELISA wells (Fig. 1). We have not tried to increase the selection stringency beyond the third round, which used a capture concentration of 2 ng/ml mAb 763.74 in the immunotubes.

In a competitive chemiluminescence phage ELISA, an increasing amount of the anti-id mAb MK2-CH γ 1 was added to a constant amount of the peptide phages. After the preincubation, the phages were added to the wells coated with mAb 763.74, and we found that the binding of the selected phage population to the paratope was

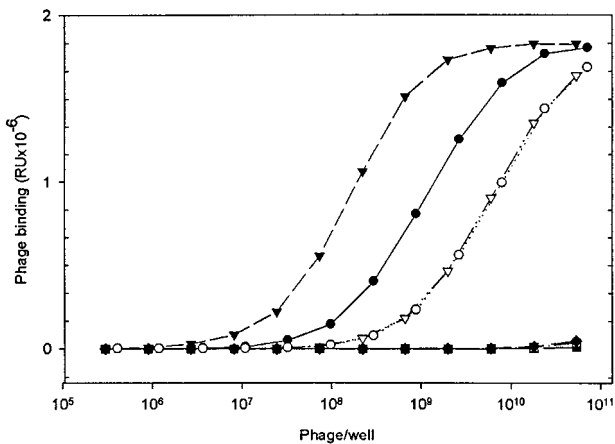


Fig. 1. Selection of phages binding to antimelanoma mAb 763.74 from a heptapeptide phage library. Microtiter plates were coated with the antibodies or BSA and incubated with the heptapeptide phage population obtained after the second (●) and third (▼) round of selection on mAb 763.74. Phages bound nonspecifically to mAb ACA125 independently of the stringency of the selection [second (○) and third (▽) round]. The phages from the third round of selection do not bind to other negative controls used in the experiments (■, BSA; □, control mAb 1; and ◆, control mAb 2). The binding phages are detected by phage ELISA in relative units (RU).

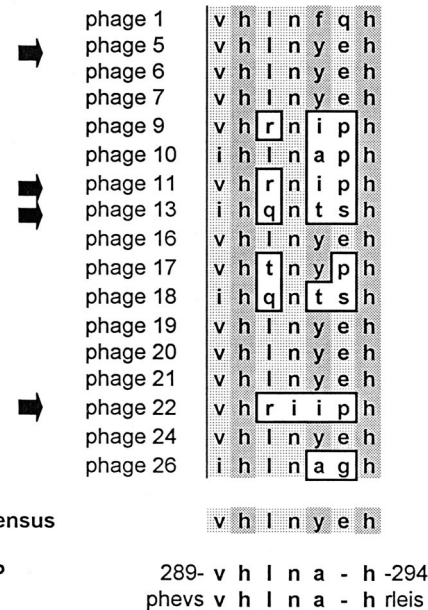


Fig. 2. Consensus aa sequence derived from the DNA sequence of individual phage clones and identification of the putative MCSP epitope recognized by mAb 763.74. The five aa up- and downstream of the MCSP epitope are also indicated. The arrows indicate the peptides subsequently synthesized and further analyzed.

inhibited by mAb MK2-CH γ 1 but not by two irrelevant isotype-matched antibodies to mAb 763.74 (data not shown). After a third and final round of selection on mAb 763.74, individual phages were propagated on *E. coli* cells, and clones were selected for DNA sequence analysis.

Identification and Characterization of the Heptapeptides Encoded by the Phage Selected on mAb 763.74. The DNA encoding the heptapeptide displayed by 18 different phages was sequenced, and the consensus sequence VHLNYEH was deduced (Fig. 2). The VHLNYEH sequence from phage 5 was found eight times, whereas the other sequences were found only once or twice. This strongly suggests that VHLNYEH represents the optimal sequence binding to mAb 763.74. The binding of phage 5 bound to mAb 763.74 was similar to the binding of the original phage population from which it was isolated (data not shown). Furthermore, it bound to the site on mAb 763.74 that is required for the binding of anti-id mAb MK2-CH γ 1 (Fig. 3A). The VHLNYEHSGSGK peptide was synthesized and biotinylated on the side chain of the lysine at the COOH terminus of the peptide. The residues SGSG were designed as a linker to allow the spatial separation of the epitope from the coupled biotin molecule. The same results were obtained in competitive ELISA experiments in which mAb 763.74 was incubated with different concentrations of the anti-id mAb MK2-CH γ 1 before addition to the biotinylated VHLNYEHSGSGK peptide coated in streptavidin-containing wells (data not shown). Melanoma A375 cells, but not the MCSP-negative cell line S7, were able to inhibit the binding of the VHLNYEH-SGSGK-(biotin) peptide to coated mAb 763.74 in a concentration-dependent manner (Fig. 3B). These results demonstrate that the antigen is a competitor for the peptide-binding site of mAb 763.74.

The aa consensus sequence derived from the selected phage library was used to scan the known aa sequence of the MCSP protein for homology. The best match was found between positions 289 and 294 of the antigen (Fig. 2). The major discrepancy was that the two His were only three residues apart within the antigen, whereas they were always separated by four residues in the selected sequences displayed by phages. Peptides corresponding to several of the selected phage peptide sequences or to the hexapeptide identified on the MCSP

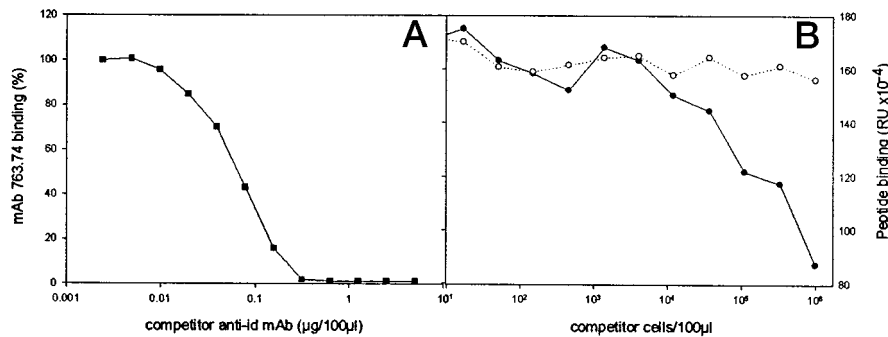


Fig. 3. Inhibition of the binding of the peptidic epitope to mAb 763.74 by anti-id mAb MK2-CH γ 1 and melanoma cells. A, mAb 763.74 was preincubated in the presence of competitor anti-id mAb MK2-CH γ 1 before addition to microtiter wells coated with the phage 5. Binding of mAb 763.74 was detected by ELISA. B, melanoma A375 (●) or S7 cells (○) were preincubated in wells coated with mAb 763.74. After washing, the VHLN γ EH-SGSGK(biotin) peptide was added to the wells and its binding to the antibody detected by ELISA with streptavidin conjugated to horseradish peroxidase. A375 cells express the MCSP antigen, whereas S7 cells do not.

antigen were synthesized. These peptides were the MCSP-derived peptide (VHINAH); its derivative VHLNAH; the peptide PHEVS-VHINAHRLAIS, which encompasses five aa upstream and downstream of the putative MCSP antigen epitope; and the peptides encoded by phages 5 (VHLN γ EH), 11, 13, and 22, (see Fig. 2 for the definition of these peptides). Clearly, all of the peptides derived from the phage library and the three peptides derived from the antigen were able to inhibit the binding of mAb 763.74 to the coated phage 5 in a concentration-dependent manner (Fig. 4). The peptides defined by phages 5, 11, 13, and 22 displayed very similar properties (results not shown). As expected, the random peptide control sequence (LQD-

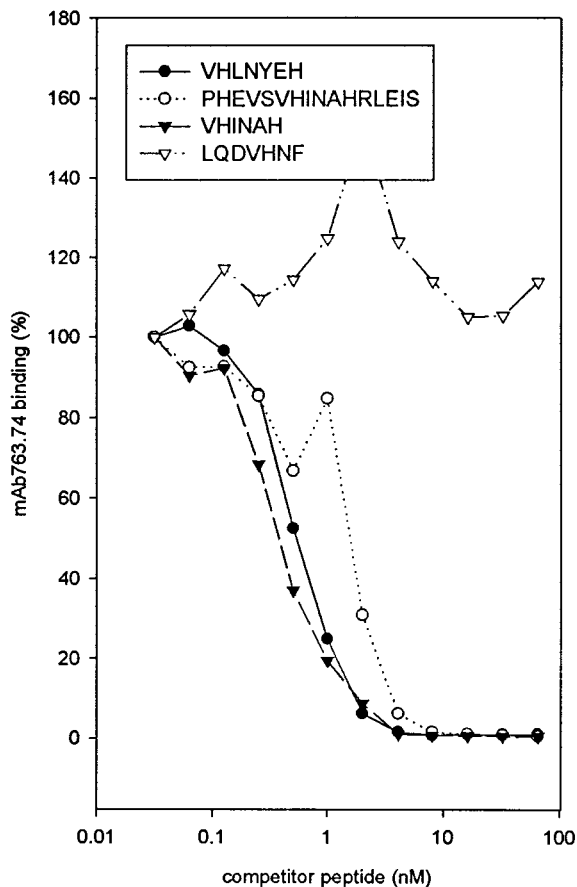


Fig. 4. Inhibition of the binding of mAb 763.74 to the peptidic epitope by soluble peptides. The microtiter plates were coated with the phage 5. mAb 763.74 was preincubated with the different peptides at the concentrations indicated and added to the wells (100 μ l/well). Binding of mAb 763.74 to phage 5 was detected by ELISA.

VHNF) did not compete. By comparing the inhibition pattern of the interactions between phage 5/mAb 763.74 and the different peptides, we found minor differences; however, we consistently observed that the VHINAH peptide inhibited the interactions better than the consensus peptide VHLN γ EH. These results demonstrate that the epitope for mAb 763.74 is VHINAH, which corresponds to the sequence between positions 289 and 294 of the MCSP antigen.

To demonstrate that the peptide identified as the epitope for mAb 763.74 can be used as a surrogate for the MCSP antigen in ELISA experiments, we immobilized the biotinylated peptide to streptavidin-coated microtiter plates and added the antibody at different concentrations in 10% human serum obtained from normal blood donors and measured the presence of the antibody by ELISA. We were able to measure the presence of the antibody down to a concentration of 40 ng/ml. The response was blocked completely by preincubation of the serum to which the antibody had been added with 20 μ g/ml of the anti-id mAb MK2-CH γ 1 (Fig. 5). Similar results were obtained by addition of the murine mAb 763.74 into a nonimmune mouse serum (results not shown).

DISCUSSION

To prove the concept of an anti-id therapeutic vaccine based on mAb MK2-23 conjugated to keyhole limpet hemocyanin (1, 11), one prerequisite is to measure the specific anti-antigen response within the general anti-anti-id response directly in the sera of immunized patients or animals. Obviously, to be of use, an immune response against the anti-id antibody should be directed against the MCSP and have a specificity similar to the anti-MCSP mAb 763.74. Hence, an assay format that uses the antigen directly, either as purified antigen or with antigen-positive cells, is needed for the evaluation of the clinical sera. Because the high-molecular weight MCSP antigen is not available in a purified state, the detection of the reaction of the specific anti-anti-id antibodies with the antigen presently is performed in technically demanding cell-based ELISAs. At the present time, the nonspecific binding of antibodies in the serum of animals or humans to melanoma cells is a serious problem in these assays. Cancer patients are known to develop antibodies against many cancer-associated antigens (32). In cell-based ELISAs, these antibodies will obscure the evaluation of the anti-anti-mAb response after vaccination. For these reasons, we decided to identify and characterize the epitope recognized by anti-MCSP mAb 763.74 from phage display libraries.

Because it is known that the antibody binds to the MCSP antigen after separation on SDS-PAGE (Refs. 1, 30, and our own results), we decided initially to select MCSP protein fragment displayed by the phages after the cloning of random DNase I-treated cDNA fragments

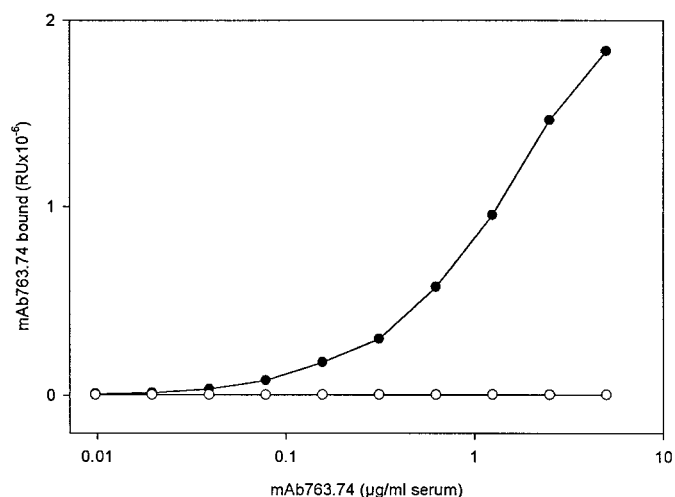


Fig. 5. Detection of mAb 763.74 added into 10% human serum. The VHLNAYEH-GSGSK(biotin) peptide was coated to streptavidin microtiter plates. mAb 763.74 was added to 10% human serum at the concentration indicated, and bound mAb was detected by ELISA with a horseradish peroxidase-conjugated antimouse antibody (●). After preincubation of the serum with 20 µg/ml of the anti-id mAb MK2-CH γ 1, the residual antibody-binding activity in the serum was also measured (○).

into a display vector. Clearly, an epitope can be identified from gene fragment libraries only if the epitope is a linear peptide. If the epitope is discontinuous or if modifications of the antigen are important elements of the epitope, these libraries are not suitable. Because this approach failed in our experiments, we reinvestigated the possibility that an oligosaccharide could be involved in the epitope for mAb 763.74. Our results indicated that the epitope is not sensitive to *N*-glycosidase F and thus is not an *N*-linked oligosaccharide. This confirms the results reported earlier by Kantor *et al.* (30), who have demonstrated that the epitope is resistant to the treatment of the melanoma cell line HO-1 with tunicamycin and *N*-glycosidase H. We still have not proven that the epitope does not incorporate an *O*-linked oligosaccharide.

Peptides have been demonstrated to be able to mimic nonlinear epitopes (15, 16, 18, 33) and nonpeptidic epitopes (34–37). We thus tried to identify peptides that mimic the mAb 763.74 epitope by using a random peptide phage display library. Using the conditions of selection defined for the screening of the gene fragment library, we immediately were able to identify specific mAb 763.74 binders. Specificity control experiments (Fig. 3) demonstrated that the peptides displayed by the phages specifically bind to the paratope of mAb 763.74. These results were confirmed with the use of the soluble synthesized peptides that were derived from the selected phages (Fig. 4). To find out whether we were dealing with a mimetic peptide that is a surrogate for a nonpeptidic epitope or with a linear peptide on the antigen, we screened carefully the aa sequence of the MCSP protein for homology with the consensus heptapeptide. As expected, the best match was identified on the extracellular domain of the antigen between positions 289 and 294. Surprisingly, we observed that the distance between the two His residues observed in the selected sequences was always four aa, and this was not found within the MCSP protein. Nevertheless, the peptide derived from the MCSP antigen binds specifically to the mAb 763.74 paratope in the competitive ELISA. These observations argue strongly that the MCSP hexapeptide is indeed the epitope for mAb 763.74.

After the removal of the signal sequence in the *E. coli* periplasm, the phages present the peptides with free NH₂ termini. It cannot be excluded that the selection might have been influenced by this free NH₂ terminus. Because the MCSP *bona fide* epitope is expected to be

found within a protein sequence, the NH₂ terminus will not be free. By adding the five aa present on both ends of the peptide identified in the MCSP sequence, we attempted to obtain a peptide that matches the original situation of the epitope in its natural environment more closely. The longer peptide indeed binds to the paratope of mAb 763.74 and inhibits the binding of the mAb to phage 5 coated to ELISA wells.

At this point we do not have any good explanations for our negative results with the selection of protein fragments displayed by the gene fragment library. We should have been able to identify the same epitope because the number of clones available for the selection was theoretically large enough to allow the cloning of the linear epitope. In summary, these results combined with the *N*-glycosidase data show that the MCSP epitope recognized by mAb 763.74 consists of a peptide sequence, without evidence for the involvement of carbohydrates.

The detection of the binding of mAb 763.74 added into human and murine sera to VHLNAYEH-SGSGK(biotin) peptide microtiter plates demonstrates that an immunosorbent assay based on the defined MCSP epitope can be an advantageous alternative and can replace the demanding cell-ELISA for the evaluation of clinical trials of an anti-id antimelanoma vaccination with the MK2-CH γ 1 anti-id mAb. In particular, the results obtained with the addition of the anti-MCSP mAb 763.74 into sera make us confident that the identified MCSP epitope peptide will be suitable for the detection of the immune responses of patients against the MCSP in clinical trial samples.

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