Epitope Specificity of the Monoclonal Anticytokeratin Antibody TS1


Abstract

Due to their abundance in epithelial cells and deposition in necrotic regions intratumorally, cytokeratins (CKs) have been established as valuable targets for both radioimmunolocalization and radioimmunotherapy. The target epitope for the monoclonal anti-CK8 antibody, TS1, used for both experimental radioimmunolocalization and radioimmunotherapy, was determined by means of synthesis of 96 overlapping peptides that covered the entire CK8 molecule. A highly conserved peptide sequence, spanning amino acids (aa) 343–357 and covering the discontinuous epitope in the helical 2B domain, was identified. The epitope retains its helical structure, as shown with circular dichroism spectroscopy, although the length of the peptide (i.e., >20 aa) is crucial for maintenance of immunoreactivity.

To determine which aa residues are crucial for binding to the monoclonal antibody, alanine scanning was performed on a 26-mer covering aa 340–365, with the sequence QRGELAIKDNANKLSELEAALQRKQK. The 26 modified peptides were evaluated using ELISA and BIAcore technology. The uniqueness of this epitope has been established by database sequence comparisons.

Introduction

CKs are interesting targets for experimental radioimmunolocalization and radioimmunotherapy because of their significant deposition in necrotic tumor regions. The availability of CKs is substantial because the low solubility of the antigens minimizes the release of antigen into the circulation and the stability of their structure entails a relative resilience to enzymatic degradation (1). These properties allow the CKs to remain in the necrotic tumor regions for substantial time periods, facilitating a significant accumulation of radiolabeled antibodies in the tumor, which is favorable for both diagnostic and therapeutic purposes (2–6).

The target availability is crucial to the optimization of the targeting procedures, and knowledge of the epitope specificity of the antibody is fundamental. Seventy % of malignant tumors are derived from epithelia and contain significant amounts of CK. Of these, a substantial proportion is derived from simple epithelia, which essentially all express CK8 (7) and, thus, can be targeted using an anti-CK8 mAb, provided the tumor contains necrotic regions (2).

CKs are members of the multigene family of IFs and are constituents of the cytoskeleton of all epithelial cells. The cytoskeleton is composed of a complex network of morphologically distinct filaments, organizing the contents of the cytoplasm (8–11). Currently, at least 20 different CKs can be distinguished (12). They constitute a family of closely related, phylogenetically conserved, distinct proteins that are biochemically and immunologically related (9, 10). CKs feature many unique characteristics among the IF proteins, including:

(a) There are two subtypes of CKs based on sequence homologies: type I keratins (CK9–CK20) are smaller (Mr 40,000–56,500) and relatively acidic, whereas type II keratins (CK1–CK8) are larger (Mr 53,000–67,000) and basic-neutral (9, 11). This dual nature has functional relevance because CK filaments form obligate heteropolymers made of type I and type II chains in a 1:1 molar ratio. The process of oligomerization leads to the formation of typical 10-nm-thick filaments.

(b) In general, CKs display a pronounced sequence diversity that is not found in other IF proteins. Among type I CKs, the α-helical domains share 50–99% sequence identity, whereas type II CKs display 30% homology in these regions (13).

The complex expression patterns of CKs are both tissue and differentiation specific, and malignant cells usually retain their CK expression pattern (9), which provides an important potential means for identifying the origin of malignant cell types, simply by characterization of their CK content. Due to the complexity of CK expression and assembly, knowledge of the target epitope-antibody interactions is a prerequisite for further improvements in targeting.

The TS1 antibody was recently characterized and determined as being specific for CK8 in the International ISOBM TD5-1 Workshop, which encompassed 30 anti-CK mAbs (14). This antibody has proved useful for both experimental radioimmunolocalization and radioimmunotherapy (5, 6, 15). The aim of this investigation was to characterize the epitope on CK8 for the mAb TS1.

Materials and Methods

mAb. The anti-CK mAb TS1 (IgGk) was obtained from InRo Biomedek (Umeå, Sweden; Ref. 16).

CK8 Peptide Mapping Using Synthesized 20-mer Peptides. The aa sequence of human CK8 was obtained from Swiss-Prot Protein Sequence Data Bank. A set of 96 peptides (20 residues long) were synthesized on a hydrophilic membrane sheet using standard Fmoc chemistry (Research Genetics, Huntsville, AL). The peptides covered the entire CK8 molecule (aa 1–483) and were shifted 5 aa from the NH2- to the COOH-terminal end.

Non-specific binding sites were blocked by immersing the membrane in 5% nonfat dried milk in PBS-T for 1 h at room temperature. The mAb TS1 at 1.2 mg/ml was diluted 1:1000 in PBS-T blocking buffer and exposed to the membrane for 1 h. After washing, bound antibody was detected using HRP-conjugated goat antimouse antibody (DAKO) diluted 1:3000 in PBS-T blocking buffer (1 h). After several washings, the spots corresponding to antibody-binding peptides were developed using luminescence activation (ECL kit; Boehringer Mannheim) and a 1-s exposure to Kodak-XAR film.

Specific coordinates to peptide numbers were easily identified because the peptides were synthesized within defined spots on the membrane in a configuration that mimics the standard 12 × 8 96-well microtiter plate array.

In a parallel investigation, the TS1 epitope was anticipated to be contained within one of three previously determined immunodominant regions on CK8. Synthetic peptides were produced corresponding to aa 67–80, 325–350, and 340–365 on rink amide MBHA resin (Novabiochem, Läügelfingen, Switzerland).
land) in the multiple peptide synthesizer SyRo (MultiSyntech, Bochum, Germany) by using Fmoc methodology (17). aa (Kebo Lab AB, Spånga, Sweden) were activated with diisopropylcarbodiimide (Sigma Chemical Co., St. Louis, MO) in the presence of hydroxybenzotriazole (Novabiochem). The purity of the peptides was analyzed by reversed-phase HPLC.

**Alanine Scanning of the Target Peptide.** Synthetic peptides were produced on a rink amide MBHA resin (Novabiochem) in the multiple peptide synthesizer SyRo (MultiSyntech) using Fmoc methodology (17). aa (Kebo Lab) were activated with diisopropylcarbodiimide (Sigma) in the presence of hydroxybenzotriazole (Novabiochem). The purity of the peptides was analyzed by reversed-phase HPLC. To map critical aa present in a reactive peptide sequence, we synthesized new substitution peptides in which each aa was sequentially replaced by alanine, except for the alanines and glycines (18). These residues were instead sequentially replaced by lysines that contain more bulky and charged side chains. HPLC analysis of randomly selected crude peptides showed main products of 33–55% purity.

**ELISA of the Alanine-substituted Peptides.** ELISAs on the modified peptides were performed by coating microtiter wells with 10 μg/ml of each of the 26 different peptides in a volume of 200 μl, followed by incubation at +8°C overnight. The ELISA plates were washed three times with ELISA wash (170 mM NaCl-0.05% Tween 20). TS1 was added in a 5 μg/ml concentration, and the plates were incubated for 3 h at room temperature. Rabbit antimouse antibody at a ratio of 1:1000 in PBS-Tween [150 mM NaCl, 10 mM Na₂HPO₄ (pH 7.4), and 0.05% Tween 20] coupled to HRP was used for detection.

**Interaction Analysis Using BIAcore 2000.** The binding between TS1 and the 26 modified peptides was compared to the binding between TS1 and the original, nonmodified target peptide using surface plasmon resonance, the BIAcore 2000 (Pharmacia Biosensor AB, Uppsala, Sweden). The molecular interactions occur on the surface of a CM 5 sensor chip, which consists of a glass slide covered with a thin gold film bound to a carboxylated matrix of the dextran. One of the reactants, i.e., the ligand, is coupled covalently to the dextran matrix, whereas one or several reactants, i.e., the analytes, are allowed to pass over the surface. Optical phenomena of surface plasmon resonance are used to detect changes in optical properties as the concentration of molecules on the surface is modified. The changes in the resonance signal are referred to as resonance units.

**CD Spectroscopy of the Target Peptide (26-mer).** CD is a powerful technique for investigating the secondary structure of peptides and proteins (19). The CD spectra obtained from proteins are primarily generated by the amide chromophore, whereas the secondary structure can be established by amide-amide interactions. CD spectroscopy was used to estimate the secondary structure of the target peptide. A Jasco J720 spectropolarimeter fitted with a temperature controller and a Quartz cuvette with 0.2-cm path length containing 400 μl of reaction mixtures were used. The CD measurements were carried out in a buffer containing 20 mM Na₂HPO₄-Na₂HPO₄ (pH 6.0). The spectrum used for secondary structure estimation was recorded at room temperature. The spectrum was collected between 190 and 250 nm in 0.5-nm steps, with a 1 s average time for each scan. From the measured CD signal, with the ellipticity \( \theta_{obs} \) at 222 nm in mdegrees, the mean residual molar ellipticity \( \theta_{222} \) (deg cm² dmol⁻¹) was calculated:

\[
[\theta]_{222} = \theta_{obs} \times 10/L \times C' 
\]

where \( L \) is the lightpath in decimeters and \( C' \) is the molar concentration of aa residues (mol/l) based on an average residue molecular mass.

**Results**

**Screening for the Target Peptide.** To localize the TS1 epitope, the set of 96 peptides, each 20 residues long with an offset of 5 aa, covering the entire CK8 molecule, were tested for antibody binding. The results are shown in Fig. 1. TS1 bound only to peptides 71 and 72, which share a 15-aa sequence corresponding to aa 343–357 on human CK8 (Table 1). In the independent, parallel investigation, the 26-mer corresponding to aa 340–365, encompassing the sequence above, was found to be the only peptide that was reactive with TS1.

**Immunoreactivity of Synthesized Peptides with Modified Sequence.** The ELISA data, using peptides with alanine substitutions, revealed two areas on the 26-mer target peptide with impaired binding to TS1 (Fig. 2), corresponding to aa 347–351 and 354–358. This indicates that aa positioned within these regions participate in the antibody binding to the epitope.

The BIAcore data showed similar results (Fig. 2) regarding the binding properties of the 26 modified peptides. There were nine peptides that displayed significantly higher \( K_c \) as compared to the original target peptide, namely, alanine substitutions at aa 347–350.
and 353–357, of which six are identical with the ELISA results. The $K_d$ of the original peptide was $1.68 \times 10^{-4}$ s$^{-1}$, whereas the $K_d$ of the nine alanine substituted peptides ranged between $2.74 \times 10^{-3}$ s$^{-1}$ and $1.25 \times 10^{-2}$ s$^{-1}$.

**CD Spectroscopy of the Target Peptide (26-mer).** Fig. 3 illustrates the CD spectra of the synthetic, 26-mer target peptide (20 µM), displaying a double minimum at 208 and 222 nm and a maximum at 195 nm, which is a typical feature for pure α-helical structures. The amount of α-helix is calculated according to the following equation:

$$\% \text{ α-helix} = \left( \frac{R - [\theta_{222}]}{R - A} \right)$$

where $A$ and $R$ are the mean residue molar ellipticities for a model α-helix ($A = -38,000$ deg-cm$^2$/dmol) and random coil ($R = 3,900$ deg-cm$^2$/dmol) peptide. The CD spectrum of the 26-mer shows a helix content of 95%. A molar ellipticity of $-35,960$ deg-cm$^2$/dmol was used in this calculation.

**Discussion**

The epitope of TS1 on CK8 is contained within aa 343–357 near the charge shift region in the helical rod domain 2B. This rod domain contains the most highly conserved sequences, and only aa substitutions that are compatible with an α-helical structure have been tolerated. The rod consists of four domains rich in α-helices, referred to as 1A, 1B, 2A, and 2B, separated from each other by three regions of β-turns. There is a distinct discontinuity near the center of segment 2B, known as the charge shift region, which is a unique property shared by all CKs (3, 7). These helical parts are relatively constant in size and contain a succession of heptad repeats or four complete turns of the keratin α-helix. aa 346, 349, 353, and 356 (asterisks) make up the contact zone between adjacent α-helices in a coiled-coil heterodimer. These aa are not exposed for antibody binding. Shaded aa, one possible epitope for TS1 based on CK specificity and binding to peptides. However, the data do not permit an exact prediction of the involved aa.

Because TS1 binds to native CK8 IFs, the epitope must be exposed in the heterodimerically assembled CK filaments, in which CK8 exists as a coiled-coil α-helical structure. If a wheel diagram is constructed according to Böttger et al. (20) and applied to CK8, the relationship of exposed aa can be visualized (Fig. 4). The diagrams show the helix end-on with the periodicity of seven residues forming two turns of an α-helix. The preferentially hydrophobic residues at first and fourth position of each heptad (Fig. 4, *asterisks*) form the interfacing surface between two dimerizing polypeptide chains. These aa will not be exposed to the aqueous environment and are, thus, not involved in antibody binding in the native filament. aa must be on the outside of the helical turns to participate in binding. Both the ELISA and the BIAcore data support the concept because the reactivity between TS1 and the modified peptides is lower than the original target peptide at the aa 347, 348, 350, 354, 355, and 357 (Fig. 2). Position 351 illustrated impaired binding using ELISA technology, which could not be verified using the BIAcore. The shaded aa in Fig. 4 give a steric presentation of the possible interactive surfaces. According to Böttger and Lane (21) and our observations, the peptide length is important when anti-CK antibodies are probed to achieve binding. The target peptide (26-mer) was also confirmed to maintain its epitope subsequently demonstrated lower reactivity (data not shown). In the case of interaction between TS1 and CK8, the epitope is discontinuous, and rather long sequences of flanking aa are necessary for the maintenance of the
α-helical structure, enabling the correct spatial epitope positioning for antibody binding.

Comparison and alignment of the target peptide (15-mer) with known proteins was performed (Swiss-Prot; Table 2), demonstrating that the identified sequence is unique for human CK8 but shows a high degree of homology with the other type II CKs. Within the chosen sequence it is interesting to note that all type II CKs have unique combinations in the four aa positions 350, 351, 354, and 355, which were demonstrated to be involved in the specific binding between TS1 and the target peptide (20-mer and 26-mer) on CK8.

By use of sequence conservation analysis, helix 2B has been established as the most variable region within the rod domain of CKs, except for the conserved helix-termination peptide. This indicates that mAbs which are selected due to their ability to discriminate between CKs may frequently be those that target epitopes in helix 2B (20). The recently reported specificity analysis of 30 anti-CK mAbs confirmed this observation (14).

Acknowledgments

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References


Table 2. Sequence homology between human CK8 and other human type II CKs

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a Hydrophobic residues that are in contact with the adjacent α-helix (i.e., not exposed to antibody binding).

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