An Epitope in Coil 2B of Cytokeratin 8

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An immunological epitope has been located at the well preserved heptade discontinuity in Coil 2B of human cytokeratin 8, with the aid of synthetic peptides, antibodies to these and monoclonal antibodies to cytokeratins. CD revealed 37 % α-helix in a 31-peptide.

The cytokeratins, the most complex class of intermediate filament (IF) proteins, have been identified in the epithelia of several species.[1–4] In humans, the 19 keratins can be grouped into the more acidic (type I) cytokeratins and the more basic (type II) cytokeratins.[1,5] The intermediate filaments in epithelial cells have been found to be constructed of heterotetramers containing two type I and two type II cytokeratin molecules.[6] The IF proteins are built according to a common motif of a central 315 AA rod portion consisting of three (four) helices connected by short non-helical sequences, an N-terminal head sequence and a more or less elaborate C-terminal tail portion.[7,8] The helices intertwine to a coiled coil conformation, which is well established by the hydrophobic heptated matrix, recognizable all through the helices, with a discontinuity appearing near the middle of Coil 2B.[9] During studies of Tissue Polypeptide Antigen (TPA), a protein widely used in serological and immunohistological monitoring of carcinomas,[10–12] partial sequences were found to show homology with IF proteins.[13–15] This observation prompted an investigation of TPA and its antibody in relation to the known cytokeratins. In immunoblotting experiments, rabbit antibodies to human TPA subfraction B1 reacted with cytokeratins 8, 18 and 19, of which 8 belongs to type II and 18 and 19 to type I. The same antibodies decorated the IF's of cells of simple epithelia and adenocarcinoma cell lines.[16]

Now that a partial structure of the human cytokeratin 18 and the complete sequences of the bovine counterparts of cytokeratins 8 and 19 have been published,[6,17,18] as well as the sequence of the human epidermal type II cytokeratin 6B,[19] and these sequences have shown great similarity[6] with cyanogen bromide fragments of TPA, some differences of immunological interest have been observed between human cytokeratin 8 represented by TPA BrCN:C, bovine cytokeratin A and human cytokeratin 6 which are presented in this paper.

Materials and methods

Antigens and antibodies. TPA subfraction B1 was prepared according to the method described by Lüning *et al.*[12] and Wiklund *et al.*,[20] and had a specific activity of 3–5 units per microgram. All activity measurements of TPA and related peptides were made with the TPA RIA prolifigen® test manufactured by AB Sangtec Medical, Bromma, Sweden. The RIA test is based on Horse anti-HeLa serum for primary antibody. Rabbit anti-TPA B1 was prepared and standardized according to Wiklund *et al.*[20] Cyanogen bromide fragments of TPA were prepared as described earlier[13] and denoted TPA:BrCN:B and TPA:BrCN:C.

Rabbit anti-peptide M255R. Five mg of M255R in 0.5 ml of water were incubated at pH 8.4 and 20 °C with 7 mg of bovine serum albumin and 20 mg of glutaraldehyde for 2 h. Sodium boro-

Table 1. Sequences of fragments and synthetic peptides.

	Sequence (positions 1–57)
TPA: BrCN:B	M A V L N D R L A Q Y L D E V R A L E A A N G – L E V L
R 64	T D L D D R L A K Y L D K V R A L E A A D G E L G V
R 118	A L L N D E L A E Y L A L N R A L E A A D G K L G V
R 128	A E Y L D L V R A L E A A D G
M 217 R	E L A Q Y L A L V R A L E A A N G R L
TPA: BrCN:C	M N R N I S R L Q A E I E G L K G Q R A S L E A A I A D A E Q R G E L A I K D A N A K L S E L E A A L Q R A K Q D M
R 107	N R N I S R L Q A E I E G L K G Q R A S L E A A I A N A E Q A
M 255 R	D A E Q R G E L A I R D A N A R L S E L E A A L Q R A K
M 269 R	A S L E A A I A D A E Q R G E L A I R D A N A R L
P 5	D A E Q R G E L A I K D A N A K L S E L E A A L Q R A K
P 11	D A E Q CiG E L A I K D A N A K L S E L E A A L Q R A K
P 13	D A E Q R G E L A I O D A N A K L S E L E A A L Q R A K

hydride was added until the yellow colour disappeared, and the conjugate was desalted on Sephadex G 25 in ammonium bicarbonate, and lyophilized. Rabbits were given 200 μg aliquots of the conjugate in Freunds complete adjuvant biweekly. Blood was drawn prior to each immunization and the serum was tested for antibody in an ELISA test using unconjugated peptide for coating.

CD spectra were measured on a Jasco J41 spectropolarimeter.

Peptides were synthesized by the solid phase technique using benzyl ester linkage to the solid support, and benzylic protective groups for trifunctional amino acids except arginine, for which tosyl was used. Glutamine and asparagine were protected with xanthydryl groups or coupled as active esters with 1-hydroxy-1*H*-benzotriazole. Boc groups were used for protection of α-amino groups, and double coupling with symmetric anhydrides was employed. All peptides were purified and desalted on Sephadex G50 or G25 columns and gave satisfactory amino acid analyses. HPLC on a reverse-phase column (C18), solvent system water/acetonitrile containing 0.05 % TFA, was used to assess the purity of the products. By these criteria peptides were more than 90 % pure. Sequences of peptides and fragments are found in Table 1.

A standard ELISA method was used with flat-bottomed microtiter plates. The antigen was coated at 10 μg ml^{-1} in 0.1 M sodium bicarbonate buffer. For primary rabbit antibodies, swine anti-rabbit horseradish peroxidase conjugate (Dako)

Table 2. Results of TPA RIA tests of fragments and synthetic peptides; values expressed in units per mg. Value for TPA taken from Ref. 12.

R64	4×10^{-2}
R107	–
R118	1×10^{-2}
M255R	4×10^{-2}
M269R	4×10^{-2}
P5	2×10^{-2}
P11	2×10^{-2}
P13	3×10^{-3}
TPA BrCN:B	10
TPA BrCN:C	10^{2}
R128	–
M217R	–
TPA	$3\text{–}5\times10^{3}$

Table 3. Inhibition of monoclonal antibodies by TPA BrCN:C.[a]

	Absorbance							
Mab	TS1	TS3	TS4	TS5	TS6	TS7	TS8	TS9
C	1.2×10^{-4}	10^{-2}	2×10^{-4}	190^{-3}	10^{-3}	10^{-3}	1.4×10^{-3}	10^{-3}
Mab	1.63	0.30	0.25	0.23	0.06	0.49	0.14	0.09
Mab + antigen	0.80	0.02	0.24	0.27	0.16	0.01	0.08	0.10

[a]Incubation of ab dilution (175 µl) with TPA BrCN:C (175 µl, 48 µg ml^{-1}) + 0.05 % Tween 40 18 h. Coating TPA B_1 2.5 µg ml^{-1}, 100 µl.

was used as secondary antibody with *o*-phenylenediamine as substrate. The absorbance was read at 489 nm.

Results

Radioimmunoassay of TPA fragments and synthetic peptides. Dose-related inhibition was obtained in a commercial RIA for TPA, used for monitoring cancer in patients, by the two TPA BrCN fragments B and C[13] and the synthetic peptides R64, R118 constructed on the basis of fragment B, and by the peptides M255R and M269R, P5, P11 and P13 constructed to match parts of fragment C. Smaller peptides, viz. R128 and M217R synthesized to match fragment B, are too short for α-helix formation and hence did not show inhibition in the RIA test; nor did one peptide, R107, matching amino acids 1–30 in fragment C (see Table 2). None of the peptides or fragments gave complete inhibition in the TPA test, indicating the presence of more than one epitope.

ELISA of TPA:BrCN:C with monoclonal antibodies to cytokeratins. In an ELISA test using purified TPA for coating, and eight monoclonal antibodies to cytokeratins TS 1 and TS 3–9, the BrCN:C fragment showed clear inhibition with the Mab's TS 1, 3 and 7 (see Table 3), which all recognize cytokeratin 8 but not cytokeratin 18.[21] This observation compared well with the high homology (83 %) between the fragment and the corresponding segment of bovine cytokeratin A6.

ELISA of rabbit antibody to the synthetic peptide M255R. Antibody dilution curves were measured in ELISA for rabbit anti-TPA B1, rabbit anti-M255R and pre-serum, using TPA B1, M255R and M269R as primary coating antigen (see Fig. 1). The antibody to the peptide not only reacts with the peptide used as antigen but also with TPA B1 and M269R. The pre-serum gave no response in the test. TPA B1 rabbit antiserum was used as a reference reacting with all three antigens.

Circular dichroism measurements on M255R. Circular dichroism spectra were measured for acidic, alkaline and neutral solutions of M255R and one solution of the peptide containing SDS. The ellipticities at 208 nm and 222 nm were used to calculate an approximate percentage of α-helix. The results are collected in Table 4.

Discussion

Previous studies of TPA (Tissue Polypeptide Antigen) showed that arginine could be reversibly blocked by cyclohexane-1,2-dione with complete loss and subsequent restoration of antigenic properties,[20] a feature shared also by the cyanogen bromide fragment C.[22] Acylation of the lysines had no effect on the antigenic properties.[20] The cyanogen bromide fragment C, located in coil 2 of cytokeratin 8[6] and the synthetic peptides M255R and M269R, but not R107, inhibit in the TPA RIA test. Antibodies to M255R bind to M269R. It is therefore reasonable to assume that an epitope, originally located in the α-helical part of cytokeratin 8 which was used to raise the antibody for the TPA test, is located in the overlapping area between the peptides M255R and M269R. In this area, three arginines are present in the two peptides. Since there was considerable doubt regarding the occurrence of lysine or arginine in the positions 37 and 42 of the fragment, peptide P5 was synthesized, being identical with M255R except for having lysine at those posi-

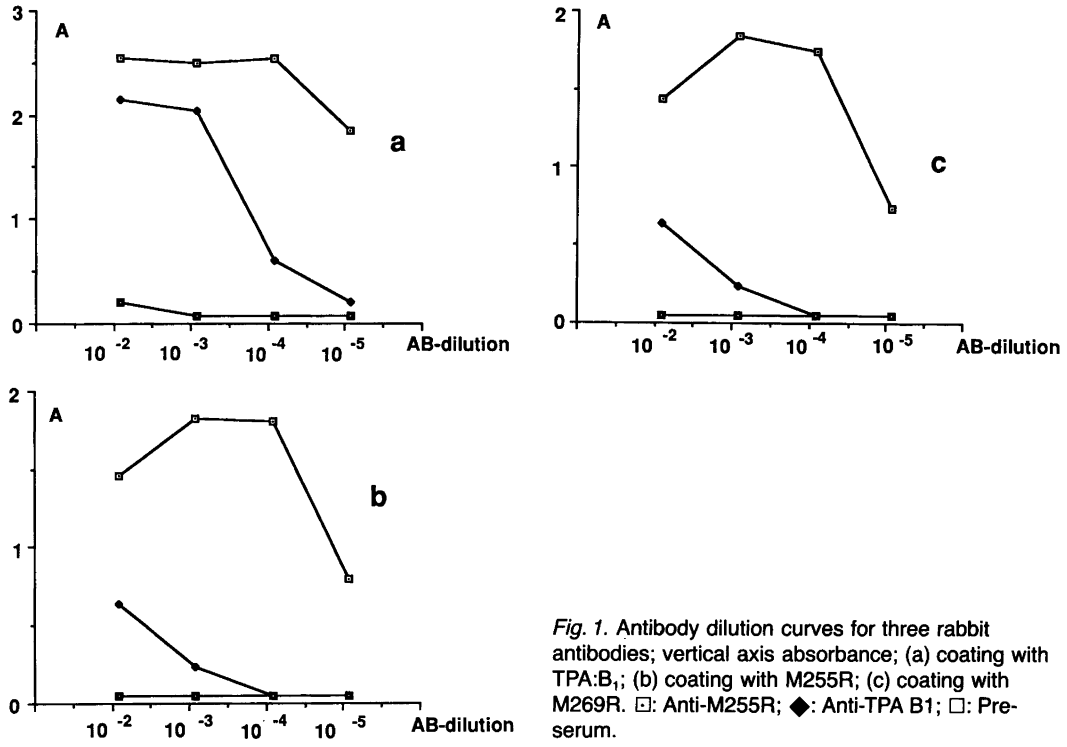

Fig. 1. Antibody dilution curves for three rabbit antibodies; vertical axis absorbance; (a) coating with TPA:B_1; (b) coating with M255R; (c) coating with M269R. ⊡: Anti-M255R; ◆: Anti-TPA B1; □: Pre-serum.

tions. This peptide is nearly as active in the TPA RIA test as M255R, restricting the area of the epitope to the vicinity of arginine 31 in the fragment, at a position corresponding to −57 in Coil 2B of bovine cytokeratin A.[6] This coil, with its heptade arrangement of hydrophobic amino acids, is part of a coiled coil arrangement in the intact cytokeratin. Substitution of citrulline for the arginine at position 31 does not change the ability to inhibit in the TPA RIA, but the substitution of ornithine for lysine 37 lowers the activity significantly.

A Chou-Fasman plot[23] for TPA:BrCN:C (see Fig. 2) shows that the area around position 31 is helical with a high probability. Circular dichroism measurements on the peptide M255R confirm the helical nature of this sequence, the 37 % α-helix being a high figure for such a short peptide. Gel filtration of the peptides M255R, P5, P11 and P13 (not presented) shows that the dimer inhibits at a lower concentration than the monomer in the RIA test, which indicates that the epitope is stabilized by the formation of a coiled coil in the synthetic peptides as well as in the protein.

Table 4. Circular dichroism values ($\Theta \times 10^{-3}$) for M255R.

Solution	Wavelength/nm			% α-Helix
	204	217	223	
0.01 M NaOH	−14750	−3450	−3600	37
0.01 M HCl	−13000	−3450	−3700	31
0.1 M $NaHCO_3$	−13800	−3910	−4025	34
0.1 M $NaHCO_3$ + 0.1 % SDS	−12400	−6000	−6000	29

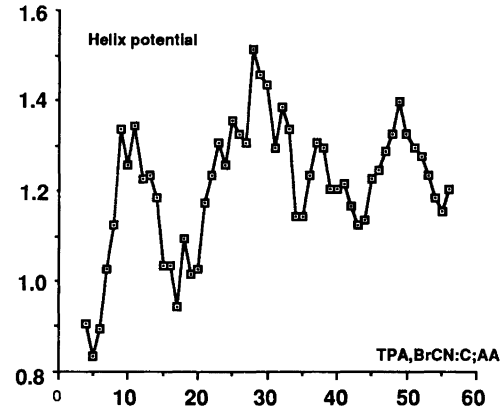

Fig. 2. Helix potential for TPA:BrCN:C according to Chou and Fasman.

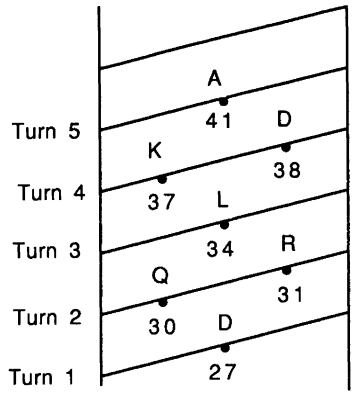

Fig. 4. Side view of the α-helix of TPA:NrCN:C with tentative epitope surface. Numbering at the amino acid signs refer to position in the fragment.

Immediately adjacent to arginine 31 is located the well preserved[9] change in polarity of the coiled coil, resulting in an octade instead of a heptade. It is reasonable to assume that the irregularity introduced should impose a higher degree of immunogenicity than the rest of the coiled coil. It is also highly probable that the outer surface of the coiled coil of the native filament should contain the immunogenic surface. The epitope must therefore contain amino acid side-chains projecting out from successive turns of the α-helix acting together. A three dimensional analysis of the neighbourhood of arginine 31 should start with Ile 25 at a position in a heptade "star" illustration and follow the helix five turns upward. This gives the diagram of Fig. 3, in which the indices at each amino acid symbol show to which turn that particular amino acid belongs. The surface of hydrophobic attraction to the other helix in the coiled coil shifts after two turns from position 1 to position 2, leaving the g position of turn 3 with arginine 31 more exposed to the aqueous medium. The outer surface of the helix comprising arginine 31 is illustrated in Fig. 4, which shows an envelope of charged amino acids surrounding a leucine with its side-chain forced into the polar medium. Just opposite the arginine at position 31, lysine 37 is located, which may explain the effect of shortening the side-chain by one carbon atom at this position. Removing the central leucine from the aqueous medium with attendant formation of a hydrophobic interaction with an antibody would account for a great deal of the free energy of binding observed as a binding constant.[10] In the epidermal type 2 cytokeratin (No. 6), as well as in bovine cytokeratin A corresponding to human cytokeratin 8, the leucine is replaced by methionine. This epitope would therefore not exist in cyanogen bromide fragments of the bovine and epidermal type II cyto-

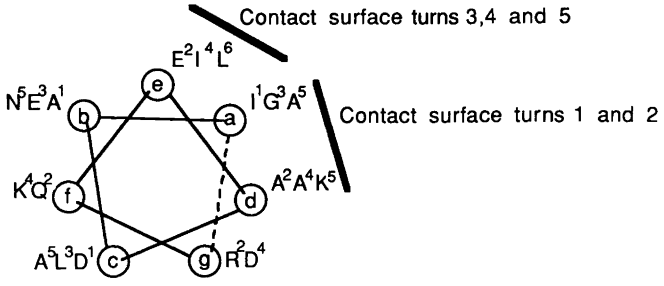

Fig. 3. Top view of five turns of the α-helix of TPA:BrCN:C. The number at each amino acid sign refers to which turn that amino acid belongs, starting with Ile 25.

keratins, and may well account for the specificity of the immunological reactions in analyses based on cytokeratin 8.

The cyanogen bromide fragment B and the N-terminal peptides derived from this fragment most certainly represent Coil 1A of cytokeratin No. 18. However, the sequence hitherto elucidated for this keratin has not yet reached the N-terminal part of the rod region, precluding a comparison. Coil 1A would therefore contain a further epitope preserved in the rod material called TPA.

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