Molecular analysis of cross-reactive human monoclonal antibody AE6F4 generated by *in vitro* immunization: Epitope mapping of AE6F4 antibody on 14-3-3 family proteins and cytokeratin 8

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Abstract

We reported previously that adenocarcinoma-reactive human monoclonal antibody AE6F4, which had been generated by *in vitro* immunization method, recognizes both 14-3-3 protein and cytokeratin 8 (CK8). In this study, to analyze the cross-reactivity of AE6F4 antibody, epitopes of AE6F4 antibody on 14-3-3 proteins and CK8 were studied by using synthetic linear peptide scanning technology. To determine the locations of B cell epitope, 48 and 95 of decapeptides covering the entire 14-3-3 proteins and CK8, respectively, were synthesized and binding to AE6F4 antibody was examined by ELISA. The AE6F4 antibody was strongly reactive to peptides containing amino acid sequences TLWTSDTQGD in 14-3-3 proteins and INFLRQLYEE in CK8. These results indicate that AE6F4 antibody can recognize the different peptide sequences in 14-3-3 proteins and CK8.

Introduction

The human monoclonal antibody AE6F4 is secreted by a human–human hybridoma line established from the *in vitro* immunization of normal human peripheral blood lymphocytes with the human lung adenocarcinoma cell line, A549 (Kawahara et al., 1992). This antibody is strongly reactive to lung cancer tissues (Shoji et al., 1994a). Shoji et al. revealed that the AE6F4 antibody recognizes a 31 kDa antigen in the cytosolic fraction of A549 cells and partial amino acid sequence of this 31 kDa antigen shows a 90% homology to the 14-3-3 protein family (Shoji et al., 1994b). Kawamoto et al. reported that the 31 kDa antigen cDNA cloned from A549 cells is identical to cytosolic phospholipase A2 (cPLA2) which is a member of the 14-3-3 protein family (Kawamoto et al., 1995).

In our previous report, we purified two new kinds of antigens (53 kDa and 40 kDa), which were neither related to 14-3-3 proteins nor 31 kDa cPLA2, in human breast adenocarcinoma cell line, MCF-7 and identified that they were cytokeratin 8 and 19 (Ichikawa et al., 1997). These data suggested that AE6F4 antibody cross-reactive to 14-3-3 proteins, CK8 and CK19. Our previous results of immunoblotting analysis with AE6F4 antibody against sodium periodate treated antigens suggested that AE6F4 antibody reacts with both carbohydrate part of 14-3-3 proteins and peptide epitopes on CK8 and CK19 (Kawamoto et al., 1995; Ichikawa et al., 1997). An accumulation of knowledge about the cross-reactivity of AE6F4 antibody is necessary in order to apply to diagnoses and medical treatments. A step toward understanding its cross-reactivity would be to determine the epitopes in antigens recognized by AE6F4 antibody. The present

Figure 1. Western blot analysis of AE6F4 binding antigens in human breast adenocarcinoma cell line MCF-7.

report therefore aims at presenting basic data on identification of epitopes recognized by AE6F4 antibody with the multi-pin synthetic peptide approach.

Materials and methods

Cells and cell culture

Human breast adenocarcinoma cell line, MCF-7 was cultured in ERDF medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂/95% air. AE6F4 antibody producing hybridomas were also cultured under the above condition.

Antibody

Culture supernatant of hybridomas containing AE6F4 antibody was used in Western blot and peptide mapping. Concentration of AE6F4 antibody was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) and adjusted to 1 $\mu\text{g ml}^{-1}$ with culture medium.

Western immunoblot

MCF-7 cell extract preparation and immunoblot analysis was performed as described previously (Ichikawa et al., 1997). Carbohydrates were cleaved by mild periodate oxidation at an acid pH, according to the method of Woodward et al. (1985). Horseradish peroxidase-conjugated goat anti-human IgM antibody (1:1000 dilution with Block Ace; Dainippon Pharmaceuticals, Japan) was used for detection.

Peptide synthesis and epitope scanning

A total of 48 and 95 decapeptides covering the entire amino acid sequences of 14-3-3 proteins and CK8, respectively, were synthesized using an epitope-Scanning Kit (Chiron Mimotopes, Australia) according to the manufacturer's instructions based on Fmoc chemistry. Peptides were designed to overlap by five amino acid residues in entire molecules (Tables 1 and 2). The success of the synthesis was monitored by simultaneous synthesis of a positive (PLAQ) and a negative (GLAQ) control peptide and by subsequently

Figure 2. Reaction of AE6F4 antibody with overlapping synthetic decapeptides of human 14-3-3 proteins.

testing their binding to the supplied monoclonal antibody. The synthesized peptides, coupled to the surface of the polypropylene pins configured to a 96-well microtiter plate, were tested for binding with AE6F4 antibody using a modified ELISA. The pins were pre-coated for 1 hr in a microtiter plate containing 2% bovine serum albumin (BSA) and 0.1% Tween 20 in PBS (PBS-T). After the pre-coating, the pins were incubated overnight at 4 °C in AE6F4 conditioned medium ($1 \mu\text{g ml}^{-1}$ IgM). They were then washed four times with PBS-T and incubated for 1 hr with alkaline phosphatase-conjugated goat anti-human IgM (1:2000 dilution with Block Ace). After four washes with PBS-T, the pins were placed in the wells of microtiter plate containing a *p*-nitrophenylphosphate substrate solution and color development was allowed to proceed for approximately 30 min at 37 °C.

Results

Reactivity of AE6F4 antibody to MCF-7 cell extract

In order to demonstrate the cross-reactivity of AE6F4 antibody, MCF-7 cell extracts were subjected to Western blotting analysis. As shown in Figure 1, the 31 kDa antigen in the soluble fraction, 53 and 40 kDa antigens in insoluble fraction were detected by AE6F4 antibody. These antigens were previously reported 14-3-3 proteins (Shoji et al., 1994a; Kawamoto et al., 1995), CK8 and CK19 (Ichikawa et al., 1997), respectively. To confirm whether carbohydrate chains

in these antigens participate in antigen recognition by AE6F4 antibody, the protein-transferred nitrocellulose membrane was treated with sodium periodate to destruct the carbohydrate chain structure. Reactivity of AE6F4 antibody with the 31 kDa antigen (14-3-3 proteins) was completely abolished (Figure 1), suggesting that carbohydrate chains play a crucial role in antigen recognition by AE6F4 antibody.

Mapping of epitopes with overlapping decapeptides of 14-3-3 proteins

Overlapping decapeptides, starting at every fifth amino acid and covering the entire 245 amino acids (aa) of human 14-3-3 proteins were synthesized on 48 pins (Table 1). ELISA was performed with AE6F4 antibody. AE6F4 reacted strongly with peptides TL-WTSDTQGD (aa 226 to 235, pin 46) (Figure 2).

Mapping of epitopes with overlapping decapeptides of CK8

Overlapping decapeptides, starting at every fifth amino acid and covering the entire 483 amino acids (aa) of human CK8 were synthesized on 95 pins (Table 2). ELISA was performed with AE6F4 antibody. AE6F4 reacted strongly with peptides IN-FLRQLYEE (aa 221 to 230, pin 45) and moderately with GLTDEINFLR (aa 216 to 225, pin 44), QYEDI-ANRSR (aa 266 to 275, pin 54) and LREYQELMN (aa 371 to 380, pin 75) (Figure 3). Pins 44 and 45 were

Figure 3. Reaction of AE6F4 antibody with overlapping synthetic decapeptides of human cytokeratin 8.

Table 1. Amino acid sequences of the synthetic overlapping decapeptides of human 14-3-3 proteins

Pin. No.	Amino acid		Pin. No.	Amino acid	
	No.	Sequences		No.	Sequences
1	1–10	MDKNELVQKA	25	121–130	MKGDYYRYLA
2	6–15	LVQKAKLAEQ	26	126–135	YRYLAEVAAG
3	11–20	KLAEQAERYD	27	131–140	EVAAGDDKKG
4	16–25	AERYDDMAAC	28	136–145	DDKKGIVDQS
5	21–30	DMAACMKSVT	29	141–150	IVDQSQQAYQ
6	26–35	MKSVTEQGAE	30	146–155	QQAYQEAFEI
7	31–40	EQGAELSNEE	31	151–160	EAFEISKKEM
8	36–45	LSNEERNLLS	32	156–165	SKKEMQPTHP
9	41–50	RNLLSVAYKN	33	161–170	QPTHPIRLGL
10	46–55	VAYKNVVGAR	34	166–175	IRLGLALNFS
11	51–60	VVGARRSSWR	35	171–180	ALNFSVFYYE
12	56–65	RSSWRVSSI	36	176–185	VFYYEILNSP
13	61–70	VVSSIEQKTE	37	181–190	ILNSPEKACS
14	66–75	EQKTEGAEEK	38	186–195	EKACSLAKTA
15	71–80	GAEKKQQMAR	39	191–200	LAKTAFDEAI
16	76–85	QQMAREYREK	40	196–205	FDEAIAELDT
17	81–90	EYREKIETEL	41	201–210	AELDTLSEES
18	86–95	IETELRDICN	42	206–215	LSEESYKDST
19	91–100	RDICNDVLSL	43	211–220	YKDSTLIMQL
20	96–105	DVLSLLEKFL	44	216–225	LIMQLLRDNL
21	101–110	LEKFLIPNAS	45	221–230	LRDNLTLWTS
22	106–115	IPNASQAESK	46	226–235	TLWTSQTQGD
23	111–120	QAESKVFYLY	47	231–240	DTQGDQAEAG
24	116–125	VFYLYKMGDY	48	236–245	EAEAGEGGEN

Table 2. Amino acid sequences of the synthetic overlapping decapeptides of human cytokeratin 8

Pin. No.	Amino acid		Pin. No.	Amino acid	
	No.	Sequences		No.	Sequences
1	1–10	MSIRVTQKSY	49	241–250	DTSVVLMDN
2	6–15	TQKSYKYSTS	50	246–255	LSMDNSRSLD
3	11–20	KVSTSGPRAF	51	251–260	SRLDMDSDII
4	16–25	GPRAFSSRSY	52	256–265	MDSIIAEVKA
5	21–30	SSRSYTSRPG	53	261–270	AEVKAQYEDI
6	26–35	TSGPGSRIS	54	266–275	QYEDIANRSR
7	31–40	SRISSSFSR	55	271–280	ANRSRAEAE
8	36–45	SSFSRVGSSN	56	276–285	AEAESMYQIK
9	41–50	VGSSNFRGGL	57	281–290	MYQIKYEELQ
10	46–55	FRGGLGGGYG	58	286–295	YEELQSLAGK
11	51–60	GGGYGGASGM	59	291–300	SLAGKHGDDL
12	56–65	GASGMGGITA	60	296–305	HGDDLPRTKT
13	61–70	GGITAVTVNQ	61	301–310	PRTKTEISEM
14	66–75	VTVNQSLSP	62	306–315	EISEMNRNIS
15	71–80	SLLSPLLEV	63	311–320	NRNISRLQAE
16	76–85	LVLEVDPNIQ	64	316–325	RLQAEIEGLK
17	81–90	DPNIQAVRTQ	65	321–330	IEGLKGQRAS
18	86–95	AVRTQEKEQI	66	326–335	GQRASLEAAI
19	91–100	EKEQIKTLNN	67	331–340	LEAAIADAEQ
20	96–105	KTLLNKFASF	68	336–345	ADAEQRGELA
21	101–110	KFASFIDKVR	69	341–350	RGELAIKDN
22	106–115	IDKVRFLQQ	70	346–355	IKDAMAKLSE
23	111–120	FLEQQNKMLE	71	351–360	AKLSELEAAL
24	116–125	NKMLETKWSL	72	356–365	LEAALQRAKQ
25	121–130	TKWSLLQQQK	73	361–370	QRAKQDMARQ
26	126–135	LQQQKTARSN	74	366–375	DMARQLREYQ
27	131–140	TARSNMDNMF	75	371–380	LREYQELMNV
28	136–145	NDNMFESTIN	76	376–385	ELMNVKLALD
29	141–150	ESTINLRRQ	77	381–390	KLALDIEIAT
30	146–155	NLRRQLETLG	78	386–395	IEIATYRKLL
31	151–160	LETLGQEKLK	79	391–400	YRKLEGEES
32	156–165	QEKLKLEAEL	80	396–405	EGEESRLESG
33	161–170	LEAELGNMQG	81	401–410	RLESGMQNMS
34	166–175	GNMQGLVEDF	82	406–415	MQNMSIHTKT
35	171–180	LVEDFKNKYE	83	411–420	IHTKTTGGYA
36	176–185	KNKYEDEINK	84	416–425	TGGYAGGLSS
37	181–190	DEINKRTEME	85	421–430	GGLSSAYGGS
38	186–195	RTEMENEFVL	86	426–435	AYGGSQAGLS
39	191–200	NEFVLIKKDV	87	431–440	QAGLSYSLGS
40	196–205	IKKDVDEAYM	88	436–445	YSLGSSFGSG
41	201–210	DEAYMNKVEL	89	441–450	SFGSGAGSSS
42	206–215	NKVELESRL	90	446–455	AGSSSFRTS
43	211–220	ESRLEGLTDE	91	451–460	FSRTSSRAV
44	216–225	GLTDEINFLR	92	456–465	SSRAVVVKKI
45	221–230	INFLRQLYEE	93	461–470	VVKKIETRDG
46	226–235	QLYEEEIREL	94	466–475	ETRDGKLVSE
47	231–240	EIRELQSQIS	95	471–480	KLVSESSDVL
48	236–245	QSQISDTSVV			

localized in coil 1B domain, pins 54 and 75 were localized in coil 2 domain. All four peptides were located in rod domain, which interacts with type II keratins, such as CK18 and CK19, when intermediate filament is organized.

Discussion

Our previous study suggested that the recognition of 14-3-3 proteins by AE6F4 antibody was affected by carbohydrate chains on 14-3-3 proteins (Kawamoto et al., 1995; Ichikawa et al., 1997). However, it was not clear, because of insufficient data, whether its epitope was carbohydrate chain or peptides. In this study, we adopted the multi-pin synthetic peptide approach to clarify whether the antigen recognition of AE6F4 antibody is affected by carbohydrate chain or not. The result of immunoblot analysis implied that AE6F4 antibody reacted with carbohydrate chains but not with protein moiety of 14-3-3 glycoproteins. However, the result of epitope mapping with multi-pin synthetic peptides system revealed that AE6F4 antibody reacted with peptide of 14-3-3 proteins (aa 226 to 235, pin 46). These results suggested that AE6F4 antibody could bind to the 14-3-3 protein derived peptide without assistance of carbohydrate chain. We think a possibility that the other region in the same 14-3-3 molecules may mask AE6F4 binding epitope when carbohydrate chains are removed from the molecule. Carbohydrate chains added on 14-3-3 proteins may play an important role in baring AE6F4 binding epitope on 14-3-3 whole molecules. In the case of synthetic pin peptides, we may not make allowance for such an obstruction by the other region of 14-3-3 molecules.

Homology between epitopes in 14-3-3 and CK8 identified by multi-pin synthetic peptide system was considerably low, contrary to expectation. Because AE6F4 antibody was generated by *in vitro* immunization method, affinity maturation of antibody accompanied with somatic hyper mutation did not occur. Indeed, variable regions of heavy and light chains of AE6F4 antibody were completely identical to germ line sequences, respectively (data not shown). Therefore the cause of the low homology among these epitopes is seemed to be the low specificity of AE6F4 antibody. In other words, AE6F4 antibody recognizes different epitopes between 14-3-3 proteins and CK8. Fortunately, 14-3-3 protein, CK8 and CK19 recognized by AE6F4 antibody were all closely related with cancer. Expressions of 14-3-3 proteins were significantly

elevated in several cancer cells and tissues (Shoji et al., 1994b; Nakanishi et al., 1997; Villaret et al., 2000). CK8 and CK19 are also known as the tumor marker proteins (Stieber et al., 1993; Xu et al., 1995). Therefore 14-3-3 proteins, CK8 and CK19 may act synergistically in the cancer cell recognition of AE6F4 antibody. In the same way, these evidences ensure the validity of adenocarcinoma specific reaction of AE6F4 antibody.

The results of the epitope mapping indicated that AE6F4 antibody was reactive to different epitopes among 14-3-3 proteins and CK8. Because of lack of affinity maturation process accompanied by somatic hyper mutation, acquisition of human monoclonal antibody with high specificity and affinity would be difficult by *in vitro* immunization method. However, if effective antigen stimulation and cloning are carried out, a useful human monoclonal antibody with considerable affinity and specificity, like AE6F4 antibody, would be obtained by *in vitro* immunization method. Therefore *in vitro* immunization method will be able to be the one of the choice of the way for the acquisition of human monoclonal antibody, as well as humanization of mouse monoclonal antibody. For more application of *in vitro* immunization, it is expected to develop the effective procedure for affinity maturation of antibody *in vitro*.

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