Identification of a surface structure in the fourth component of human complement, C4, which becomes hidden upon activation by C1s

Shinji MAEDA,*§ Yuji TAKAMARU,† Jun FUKATSU‡ and Shigeharu NAGASAWA*||
* Faculty of Pharmaceutical Sciences and † Department of Psychiatry and Neurology, School of Medicine, Hokkaido University, Kita-ku, Sapporo 060, and ‡ Department of Psychiatry and Neurology, Sapporo Medical School, Chuo-ku, Sapporo 060, Japan

Treatment of complement component C4 with C1s and methyamine induces a series of conformation changes such as to generate functional binding sites. A monoclonal antibody (mAb), Al 121/6, which does not inhibit the haemolytic activity of C4 was found to bind to native C4 and C4d, but not to C4b and methyamine-treated C4, unless these C4 derivatives were denatured. These results suggested that a linear epitope for mAb Al 121/6 in the C4d domain is originally located at the surface of C4 and becomes hidden as a result of conformational changes induced by C1s or methyamine treatment. The hidden linear epitope was exposed again upon further cleavage of C4b into C4c and C4d. Trypsin digestion of C4d and its chemical modification with phthalic anhydride suggested that the epitope is located at the C-terminal 13 kDa region of C4d and that lysine residues are involved in the epitope. There is a single lysine residue at 1259 in the 13 kDa C-terminal side of C4d and the synthetic undecapeptide Leu1244-Asp1254 was found to inhibit the binding of C4 to mAb Al 121/6, suggesting that the epitope for mAb Al 121/6 is involved in the sequence. The N-terminal portion of the peptide is partly overlapping, with a highly hydrophobic amino acid sequence spanning residues Ala1249-Leu-Leu-His-Leu-Leu-Leu1255. The surface hydrophobicity of C4 has been reported to decrease upon treatment with C1s and methyamine. So it appears that the hydrophobic sequence spanning Ala1249-Leu1255 may be hidden, together with the linear epitope, into the inner region of C4 upon treatment with C1s and methyamine.

INTRODUCTION

The complement system plays an important role in the immune defence against infection. It consists of a group of plasma and membrane proteins that, when activated by immune complexes and foreign materials, interacts in cascade fashion to accelerate the elimination of foreign materials from the body (Lachmann and Hughes-Jones, 1985; Müller-Eberhard, 1988). These effector functions of the complement system are mediated by a serine proteinase, C3 convertase, which is generated via two independent cascades, termed the classical and alternative pathways. The C3 convertase of the classical pathway is assembled from two complement components, C4 and C2, by the action of C1s (Vogt et al., 1982; Maeda and Nagasawa, 1990).

C4 consists of three non-identical disulphide-linked chains designated α, β and γ, having molecular masses of 93, 78 and 33 kDa respectively (Schreiber & Müller-Eberhard, 1974). The cleavage of the α chain by C1s produced a low-molecular-mass activation peptide, C4a and a major cleavage fragment, C4b (Schreiber & Müller-Eberhard, 1974). The cleavage reaction is accompanied by the generation of at least three binding sites, i.e. one labile and two stable binding sites on the nascent C4b. The labile binding site is the site through which a nascent C4b binds covalently on immune complexes (Gorski and Howard, 1980; Law et al., 1981). The stable binding sites are the sites for C2 and C4bp, these two components being respectively involved in the assembly and regulation of the classical-pathway C3 convertase; C4b–C2 complex is cleaved by C1s to assemble C3 convertase, C4b2a (Müller-Eberhard et al., 1967; Kerr, 1980), whereas the C4b–C4bp complex is cleaved by Factor I into functionally inactive C4c and C4d (Gigli et al., 1979; Nagasawa et al., 1980).

Although the labile binding site has been proved to be Gin994, forming an internal thioester linkage with a Cys993 (Campbell et al., 1981; Harrison et al., 1981), the sequence and location of the stable binding sites have not yet been determined.

These binding sites in C4b are generated by a series of conformational changes triggered by the limited proteolysis of C4. It was reported that C4 activation with C1s was accompanied by decrease in the surface hydrophobicity and alteration of backbone conformation (Isenman & Kells, 1982). In addition, inactive C4 having the similar conformation to C4b is known to be produced upon treatment with methyamine (Isenman & Kells, 1982). However, it has not been shown clearly which portions in a C4 molecule are conformationally labile.

Monoclonal antibodies (mAbs) which recognize neoantigenic epitopes expressed in activated complement components have shown to be effective as probes to investigate the molecular changes associated with the activation of complement components (Nilsson & Nilsson, 1982; Lambiris et al., 1985; Becherer et al., 1992). We have reported an mAb, mAb242, which recognizes a conformation-dependent neoantigen generated in C4b and inhibits the binding of C2 and C4bp to C4b (Ichihara et al., 1986). Here we examine the location of the epitope for a new mAb to C4 which binds to C4, but not to C4b, unless it is denatured. We show that the epitope appears to be located in the sequence spanning from Leu1244 to Asp1254 in C4d and is shifted from the surface into the inner region upon treatment of C4 with C1s and methyamine. There is a highly hydrophobic sequence...
adjacent to the N-terminal side of the linear epitope, suggesting that this hydrophobic sequence is also hidden, resulting in a decrease in the surface hydrophobicity.

**MATERIALS AND METHODS**

**Materials**

A hybridoma producing an mAb to C4, Al 121/6, was cloned from hybridomas that were produced with human amyloid plaque core of Alzheimer’s disease as antigens (details are available from S. N. on request). C4 (Nagasawa et al., 1980), Cls (Takahashi et al., 1975), Factor I (Nagasawa et al., 1980) and C4bp (Nagasawa et al., 1982) were purified from human plasma by the methods cited. Methylamine-treated C4 was prepared by treatment with 1 M methylamine hydrochloride at 37 °C for 60 min (Isenmann and Kells, 1982) and dialysed against 20 mM Tris/HCl, pH 7.4. C4c and C4d were produced by cleavage of C4b with C4bp and Factor I (Nagasawa et al., 1980) and purified by size-exclusion h.p.l.c. The synthetic undecapeptide Leu-Leu-His-Glu-Lys-Ala-Glu-Met-Ala-Asp was prepared by using an Applied Biosystem 430A synthesizer by the standard solid-phase procedure of Merrifield (1963). The peptide was cleaved from the resin with anhydrous HF and purified by reverse-phase h.p.l.c. on a C18 column using a 10–80 % acetonitrile gradient containing 0.1 % trifluoroacetic acid. Trypsin was obtained from Sigma. Chemicals used were of analytical grade.

**Electrophoresis and Western blotting**

Disc native PAGE was performed with 5 % polyacrylamide gels by the method of Davis (1964). SDS/PAGE was performed by the method of Laemmli (1970). Transfer of proteins to a nitrocellulose membrane and detection of antigenic determinants were performed by the method of Towbin et al. (1979). The immunoactivity of the mAb with the transfured polypeptide chains was tested using a 1:2000 dilution of polyclonal anti-C4 antisera (Capel) and PBS or Tween 20. The anti-C4-coated wells were then incubated with 50 μl of a test sample, which was prepared by mixing C4 (125 μg/ml) and the mAb (500 μg/ml) in the presence of various concentrations of a synthetic peptide. The wells were then washed and developed with the peroxidase-conjugated goat anti-mouse IgG for 2 h at room temperature. Finally, the wells were washed and developed with the peroxidase-substrate 2,2'-azobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt at 37 °C until a satisfactory colour development had occurred. The colour intensity was read at 405 nm with an e.l.i.s.a. reader (Bio-Rad).

**Haemolytic assay**

The effect of the mAb to the haemolytic activity of human serum was determined with sheep erythrocytes sensitized with IgM antibody (EA), which were prepared according to standard techniques (Mayer, 1961). Haemolysis was evaluated by measuring the absorbance of the supernatant at 541 nm.

**Size-exclusion h.p.l.c.**

This was performed with either a column of TSK G3000SW (Tosoh) equilibrated with 0.1 M phosphate buffer, pH 6.8, or Superose 6 (Pharmacia) equilibrated with phosphate-buffered saline (PBS). Elution of proteins was monitored by measuring absorbance at 280 nm.

**C4d cleavage by trypsin**

C4d was incubated with trypsin at a C4d/trypsin ratio of 50:1 (w/w) for 0.5–16 h at 37 °C. The digests were mixed with an equal volume of 1 % mercaptoethanol/10 % (w/v) SDS, boiled for 5 min, and subjected to SDS/PAGE.

**Sequence determination**

C4d fragments separated by SDS/PAGE were blotted on to an Immobilon membrane and stained with Coomassie Blue. The stained bands were excised from the membrane and directly applied to a 470 gas-phase automatic sequenator (Applied Biosystems). Phenylthiohydantoin derivatives of amino acids were determined by on-line reverse-phase h.p.l.c.

**Chemical modification of C4d with phthalic anhydride**

Phthalation of C4d was performed by the method of Pechere and Bertrand (1977) with minor modification. To C4d in 40 mM sodium carbonate/4 M guanidine, pH 8.5, was added an aliquot of 1.7 M phthalic anhydride/dioxan solution, corresponding to a 2-fold molar excess over the number of free amino groups in C4d. After vigorous stirring for 10 min at 20 °C, four further aliquots were added over an approx. 1 hr period. To the reaction mixture was then added hydroxylamine hydrochloride to 12 mg/ml at pH 9.0, kept for 20 h, and finally dialysed against PBS.

**E.I.I.s.a. for detecting binding of a synthetic peptide to mAb Al 121/6**

This was performed by the method of Engvall and Perlmann (1972) with slight modification. A 50 μl portion of a 1:200 dilution of polyclonal anti-C4 antisera (Capel) were added to each well of a 96-well microtitre plate and incubated overnight at 4 °C. The wells were blocked with 1 % BSA and washed three times with 200 μl of PBS containing 0.05 % Tween 20. The anti-C4-coated wells were then incubated with 50 μl of a test sample, which was prepared by mixing C4 (125 μg/ml) and the mAb (500 μg/ml) in the presence of various concentrations of a synthetic peptide. The wells were then washed and developed with the peroxidase-substrate 2,2'-azobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt at 37 °C until a satisfactory colour development had occurred. The colour intensity was read at 405 nm with an e.l.i.s.a. reader (Bio-Rad).

**RESULTS**

**Binding specificity of mAb Al 121/6**

Reduced and non-reduced C4 and C4 fragments were separated by SDS/PAGE. The gels were either stained in Coomassie Blue (Figure 1a) or were subjected to Western blotting (Figure 1b). The mAb bound to C4, C4b and C4d, indicating that the epitope for mAb Al 121/6 is located in C4d domain. Evidence that mAb Al 121/6 binds to reduced α-chain and C4d indicates that the mAb recognizes a linear epitope in C4d.

Next we examined the binding of mAb Al 121/6 to C4, C4b and C4d in the absence of denaturing agents. C4 or C4b was mixed with mAb Al 121/6 and separated by native disc PAGE. As shown in Figure 2, the band of C4 disappeared and a new band with a low mobility was formed when C4 was mixed with mAb Al 121/6, indicating formation of a C4–mAb complex. On the other hand, new bands were not detected in the mixture of C4b and the mAb, suggesting that mAb Al 121/6 is not accessible to its epitope in C4b unless the conformation of C4b is disrupted.

The thioester linkage of C4 is known to be also cleaved by treatment of C4 with methylamine, and the methylamine-treated C4 is functionally and conformationally similar to C4b (Janatova & Tack, 1981). To show that the conformational change neighbouring the epitope for mAb Al 121/6 is triggered by the cleavage of the thioester bond of C4, methylamine-treated C4
was mixed with mAb Al 121/6 and subjected to native disc PAGE. As shown in Figure 2, the methylamine-treated C4 failed to form a complex with mAb Al 121/6.

To ascertain that mAb Al 121/6 is capable of binding to C4, but not to intact C4b, mAb Al 121/6 was mixed with either C4 or C4b and separated by size-exclusion h.p.l.c. A high-molecular-mass immune complex formed upon mixing of mAb Al 121/6 with C4, but not with C4b, in the presence of excess mAb 121/6 (Figure 3). Thus it appears likely that the epitope for mAb Al 121/6 is located at the surface of C4 molecule and shifted into the inner portion by a series of conformational changes when the thioester bond is cleaved upon enzymic or chemical treatment of C4.

To ascertain whether the hidden epitope for mAb Al 121/6 might be exposed again in C4d, mAb Al 121/6 and C4d were mixed and subjected to size-exclusion h.p.l.c. As shown in Figure 4, C4d formed a high-molecular-mass immune complex with mAb Al 121/6.

Effect of mAb Al 121/6 on the functions of C4

We examined whether the epitope for mAb Al 121/6 is responsible for the functions of C4. First, the C4-mAb complex was isolated by h.p.l.c. using Superose 6 and allowed to react with C1s. SDS/PAGE of the reduced sample revealed that the α-chain (93 kDa) was cleaved to give an α-chain (87 kDa), indicating that binding of mAb Al 121/6 to C4 does not inhibit the C1s-catalysed α-chain cleavage (Figure 5). This result also suggests that the epitope for mAb Al 121/6 is located far from the C1s-susceptible bond Arg737-Ala738 in the α-chain of C4. Next we tested the effect of mAb Al 121/6 on the haemolytic activity of C4. Figure 6 shows the effects of mAb Al 121/6 and mAb 242 on the haemolysis of EA cells by human serum. As reported previously (Ichihara et al., 1986), mAb 242, whose epitope is closely related with the C2-binding site in C4b, inhibited the haemolytic activity of human serum, but mAb Al 121/6 did not inhibit the haemolysis of EA cells by human serum.

Production of the epitope-containing fragments by trypsin digestion of C4d

To characterize the location of the epitope for mAb Al 121/6 in C4d we attempted to prepare an epitope-containing fragment by trypsin digestion of C4d. SDS/PAGE and Coomassie Blue staining of trypsin digest of C4d showed that C4d (48 kDa) was first cleaved into a 41 kDa fragment and subsequently into a 28 kDa fragment (Figure 7). Western-blot analysis showed that the epitope for mAb Al 121/6 remained in the 41 kDa fragment, but not in the 28 kDa fragment, suggesting that the linear epitope is either released as a small peptide or destroyed during the tryptic cleavage of the 41 kDa intermediate into the 28 kDa fragment.
C4 or C4b was mixed with mAb Al 121/6 for 10 min at 37 °C and subjected to h.p.l.c. with a column of TSK G3000SW equilibrated with 0.1 M phosphate buffer, pH 6.8. The flow rate was 0.5 ml/min. (a) C4; (b) mAb Al 121/6; (c) a mixture of C4 (10 μg) and mAb Al 121/6 (5 μg) in 50 μl of 0.1 M phosphate buffer, pH 6.8; (d) a mixture of C4b (5 μg) and mAb Al 121/6 (10 μg) in 50 μl of 0.1 M phosphate buffer, pH 6.8. The arrowhead indicates the elution of the C4-mAb complex.

Figure 3 Analysis of binding of mAb Al 121/6 to C4 and C4b by size-exclusion h.p.l.c.

A mixture of C4d and mAb Al 121/6 was kept for 10 min at 37 °C and subjected to h.p.l.c. with a column of TSK G3000SW. (a) C4d; (b) C4d (10 μg) and mAb Al 121/6 (5 μg) in 50 μl of 0.1 M phosphate buffer, pH 6.8. The arrowhead indicates the elution of the C4d-mAb complex.

Figure 4 Analysis of binding of mAb Al 121/6 to C4d

About 50 μg of the immune complex isolated by h.p.l.c. was incubated with 0.15 μg of C1s for 60 min at 37 °C. The C1s-treated immune complex was reduced and subjected to SDS/PAGE with a 10% polyacrylamide gel. Lane 1, C4-mAb Al 121/6 complex; lane 2, C1s-treated immune complex; lane 3, C4b; lane 4, mAb Al 121/6. α, β, and γ are the polypeptide chains of C4. α' is the cleaved chain of C4b. H and L are the heavy and light chains of mAb.

Figure 5 Cleavage of C4-mAb Al 121/6 complex by C1s

Figure 6 Effect of mAb Al 121/6 on the haemolytic activity of human serum

Indicated volumes of diluted human serum (81-fold) were mixed with 10 μg of mAb Al 121/6 or mAb 242 as a positive control and adjusted to final 1.5 ml with gelatin/Veronal buffer. After incubation for 5 min at 37 °C, the haemolytic activity of mAb-treated serum was determined with EA cells. ■: mAb 242, ◆: mAb Al 121/6, □: control.

N-terminal side of the epitope-containing 41 kDa C4d fragment. It appears likely, therefore, that the epitope for mAb Al 121/6 is located in the C-terminal 13 kDa region released from the 41 kDa fragment.

Localization of the epitope for mAb Al 121/6 in the linear sequence of C4d

To obtain information on the amino acid residues in the epitope for mAb Al 121/6, C4d was subjected to chemical modification and tested for the binding to mAb Al 121/6 by Western blotting. We observed that mAb Al 121/6 did not bind to phthalic anhydride-treated C4d (results not shown). In the C-terminal 13 kDa region of C4d, in which the epitope is supposed to be located, a single lysine residue is present in the sequence of Leu-
Figure 7 SDS/PAGE and Western-blot analysis of trypsin-treated C4d

C4d was digested with trypsin at a C4d/enzyme weight ratio of 50:1 for 0 (1), 0.5 (2), 1 (3), 2 (4), 5 (5), and 16 (6) h at 37 °C and subjected to SDS/PAGE (10% polyacrylamide). Tryptic fragments of C4d were detected by Coomassie Blue staining (a) or Western blotting (b). Abbreviations: 41KF and 28KF, C4d fragments of 41 and 28 kDa respectively.

Figure 8 Inhibition of binding of C4 to mAb A1 121/6 by the synthetic peptide

Equal volumes of C4 (125 ng/ml) and mAb A1 121/6 (500 ng/ml) were mixed in the presence of various concentrations of the synthetic peptide for 2 h at 37 °C. Aliquots of 50 μl of the mixtures were then added to anti-C4 coated 96-well microwell plates and binding of C4–mAb A1 121/6 complex to antibody-coated plates using anti-mouse IgG was determined as described in the Materials and methods section.

Leu-His-Glu-Gly-Lys1259-Ala-Glu-Met-Ala-Asp. After this, an undecapeptide, Leu1254–Asp1264 was synthesized, and binding of the peptide to mAb A1 121/6 was indirectly tested by measuring inhibition of binding of C4 to mAb A1 121/6. As shown in Figure 8, binding of C4 to mAb A1 121/6 was inhibited in parallel with an increase of the amount of the synthetic peptide.

**DISCUSSION**

The conformational changes accompanying the C1s-catalysed C4 activation or methylamine-induced C4 inactivation have been observed by using spectral and solution scattering techniques (Isemann and Kells, 1982; Perkins et al., 1990). However, little information has been available concerning the structural regions involved in the conformational changes occurring upon C4 activation/inactivation. The present paper is the first, to our knowledge, demonstrating a surface region which becomes hidden upon C4 activation/inactivation. The conformationally labile surface region was identified as the epitope for mAb A1 121/6, which is one of mAbs against Alzheimer’s-disease amyloid proteins. Production of anti-C4 antibody by immunization with Alzheimer’s-disease amyloid proteins supported the recent reports on activation of the classical pathway of complement in brain of Alzheimer’s-disease patients (McGeer et al., 1989; Yamada et al., 1990).

Tryptic cleavage and chemical modification of C4d suggested that the Leu1254–Asp1264 would be involved in the linear epitope. The synthetic undecapeptide spanning residues Leu1254–Asp1264 competitively inhibited the binding of C4 to mAb A1 121/6, proving that the linear epitope is involved in the sequence. The amino acid sequence of the peptide spanning residues Leu1254–Asp1264 is different from the corresponding region of mouse C4 in the three residues His1254, Ala1256, and Glu1259 (Nonaka et al., 1985). These residues may act as the antigenic determinants for the production of mAb A1 121/6.

Evidence that mAb A1 121/6 fails to inhibit the functional activities of C4 suggests that the epitope region neither constitutes the functional sites of activated C4b nor locates in the vicinity of the functional sites. Interestingly enough, the N-terminal portion of this epitope is a part of long hydrophobic regions such as Alas1249–Leu-Leu-His-Leu-Leu-Leu1255. It is well known that the surface hydrophobicity of C4 decreases upon activation/inactivation of C4 (Isemann and Kells, 1982), so it seems probable that a long region spanning residues Ala1249–Asp1264 is hidden from the surface upon activation/inactivation of C4. This conformational change appears to be triggered by the split of an internal thioester linkage, which is located at approx. 100 amino acids on the N-terminal side of the epitope. There are two functionally related regions between the thioester linkage and the epitope region. One is the region comprising residues 1108–1115, which defines the specificity of surface binding of nascent C4b (Carroll et al., 1990). The other is the Ser1217, which is the acceptor site for a nascent C3b in the conversion of the C3 convertase into the C5 convertase, C4b2a3b (Kim et al., 1992). Thus it is tempting to speculate that the enclosure of the epitope-containing hydrophobic region may play a role in locking the functional sites in the proper configuration.

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**REFERENCES**


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