

# Conformational differences between surface-bound and fluid-phase complement-component-C3 fragments

## Epitope mapping by cDNA expression

Bo NILSSON,\*§ Dario GROSSBERGER,†|| Kristina NILSSON EKDAHL,\* Patricia RIEGERT,† David J. BECHERER,†¶|| Ulf R. NILSSON\* and John D. LAMBRIS†‡

\*Department of Clinical Immunology and Transfusion Medicine, University Hospital, S-751 85 Uppsala, Sweden,

†Basel Institute for Immunology, 487 Grenzacherstrasse, CH-4005 Basel, Switzerland, and ‡Protein Chemistry Laboratory,

Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

In previous studies a subset of complement-component-C3 (C3) epitopes, C3(D), expressed in denatured and surface-bound C3 and C3 fragments, has been described. These epitopes were detected by antibodies raised against denatured C3. In the present study we used a cDNA expression strategy to localize epitopes recognized by monoclonal and polyclonal anti-C3(D) antibodies. First, DNAase I digestion of C3 cDNA was used to generate 200–300 bp fragments. These cDNA fragments were expressed as  $\beta$ -galactosidase–C3 fusion proteins using the  $\lambda$ gt11 vector. The fusion proteins were tested by Western-blot analysis for reactivity with monoclonal and polyclonal anti-C3 antibodies, and the location of the epitopes were determined by sequencing the cDNA fragments. Affinity-purified polyclonal anti-C3(D) antibodies specific for denatured C3 reacted strongly with the C3 fusion fragments corresponding to segments of the 40 kDa subunit of C3c (residues 1477–1510) and the C3d fragment (residues 1117–1155 and 1234–1294) of C3. Adsorption of the polyclonal antibodies with a mixture of EAC3b and EAC3bi (degradation fragments of C3 bound to sheep erythrocytes) abolished binding to fusion proteins spanning the C3d region, but not the 40 kDa fragment of C3c. No effect was seen with the corresponding soluble C3 fragments. The monoclonal anti-C3(D) antibodies (mAbs) 7D326.1 and 7D331.1, specific for EAC3b and EAC3bi, bound to a fusion protein corresponding to amino acid residues 1312–1404, whereas mAb 7D9.2, specific for EAC3d, reacted with a fusion protein spanning amino acid residues 1082–1118. mAbs 4SD11.1 and 4SD18.1, which did not bind to any physiological C3 fragment, detected a fusion protein covering residues 1477–1510. In summary, the segments of C3 represented by amino acid residues 1082–1118, 1117–1155, 1234–1294 and 1312–1404 accommodate C3(D) epitopes that are expressed by erythrocyte-bound C3 fragments, but not by the corresponding fluid-phase fragment, whereas the segments spanning residues 973–1026 and 1477–1510 contain C3(D) epitopes that are exposed exclusively in denatured C3 and therefore hidden in physiological fragments of the protein.

## INTRODUCTION

Complement component C3 comprises two polypeptide chains (110 kDa and 75 kDa) linked by one disulphide bond and non-covalent forces (Matsuda *et al.*, 1985; Janatova, 1986). Cleavage of C3 between residues Arg<sup>726</sup> and Ser<sup>727</sup> of the  $\alpha$ -chain, by either the classical- or the alternative-pathway C3 convertase, generates the C3a and the C3b fragments. The nascent C3b molecule can bind to target molecules by either an ester or an amide bond (Law *et al.*, 1980; Gadd & Reid, 1981). The cleavage of the  $\alpha$ -chain of C3b by Factor I, first between residues Arg<sup>1281</sup> and Ser<sup>1282</sup> and secondly between residues Arg<sup>1298</sup> and Ser<sup>1299</sup>, liberates the 2 kDa C3f fragment and yields iC3b (Lachmann *et al.*, 1982). Further Factor I cleavages, essentially between residues Arg<sup>932</sup> and Glu<sup>933</sup>, generate the C3d,g fragment with the concomitant liberation of C3c (Medof *et al.*, 1982; Ross *et al.*, 1982; Nilsson Ek Dahl *et al.*, 1990).

The exposure of binding sites for complement components and complement receptors on the different C3 fragments is the result of a series of conformational changes that occurs in the molecule

upon transition from one form to another during the proteolytic activation/inactivation. These changes have been detected by both spectroscopic and solution scattering analyses of soluble C3 fragments (Molenaar *et al.*, 1975; Isenman & Cooper, 1981; Isenman, 1983; Perkins & Sim, 1986) and by monoclonal antibodies against neoantigens exposed specifically by C3 fragments (Alsenz *et al.*, 1990). Besides the conformational changes that have been observed during the degradation of fluid-phase C3 and its soluble fragments, further conformational changes occur when the C3 fragments bind to target surfaces. These changes are evident from the ability of C3 binding proteins to bind with higher affinity to surface-bound compared with fluid-phase C3 fragments (DiScipio, 1981; Pangburn & Müller-Eberhard, 1983; Ross & Medof, 1985; Becherer & Lambris, 1988) and from exposure of neoantigenic epitopes detected by monoclonal and polyclonal antibodies specific for bound C3 fragments (Nilsson & Nilsson, 1982; Aguado *et al.*, 1985; Nilsson *et al.*, 1987; Alsenz *et al.*, 1990).

Although much work has been directed toward understanding the structural features involved in the different functions of C3

Abbreviations used: C3, the third component of complement; C3b, iC3b, C3c, C3d,g, C3g and C3d, degradation fragments of C3; C3(D), antigens expressed by SDS-denatured C3, but not by native C3; DTT, dithiothreitol; DEAE, diethylaminoethyl; EAC3b, EAC3bi and EAC3d; C3b, iC3b and C3d bound to sheep erythrocytes; IPTG, isopropyl thio- $\beta$ -D-galactoside; LB, Luria-Bertani medium; PMSF, phenylmethanesulphonyl fluoride; mAbs, monoclonal antibodies; HRP, horseradish peroxidase; PBS, phosphate-buffered saline.

§ To whom correspondence should be sent.

|| Present address: Department of Cell Biology, Stanford University, Stanford, CA 94305, U.S.A.

¶ Present address: Glaxo Inc., 5 Moore Drive V-290, Research Triangle Park, NC 27709, U.S.A.

(Becherer *et al.*, 1990; Lambris, 1988), comparatively little attention has been given to the conformational changes occurring upon activation/inactivation of C3 and to the specific regions involved in these changes. In the present study we prepared monoclonal (mAb) and polyclonal antibodies with the specificity for a subset of C3 epitopes, C3(D), which are expressed on (a) surface-bound, but not fluid-phase fragments of C3 and/or (b) denatured, but not native, C3 fragments (Nilsson *et al.*, 1980; Nilsson & Nilsson, 1982, 1985, 1986). We determined the location of the recognized epitopes on C3 by generating cDNA fragments encoding different regions of the  $\alpha$ -chain of C3 and by determining which of the cDNA fragments that encode the epitopes when expressed in bacteria as  $\beta$ -galactosidase-C3 fusion proteins. The C3 regions identified by this analysis are important in establishing the distinct conformational changes that occur in C3 upon its degradation and fixation to cell surfaces.

## MATERIALS AND METHODS

### Materials

Trypsin was purchased from Worthington. Sephadex G-100, *EcoRI* linker 10-mers, CNBr-activated Sepharose and Protein A-Sepharose were obtained from Pharmacia AB. Anti-rabbit and anti-mouse immunoglobulins conjugated with horseradish peroxidase (HRP) were purchased from DAKO immunoglobulins. Microtitre plates (Immunoplates IIF) were obtained from Nunc. Bio-Gel A15m, nitrocellulose membranes and Enzymobeads were from Bio-Rad Laboratories. Carrier-free Na<sup>125</sup>I and streptavidin-biotinylated HRP complex were purchased from Amersham International. Biotin-coupled goat anti-rabbit Ig and complex of avidin/peroxidase-coupled biotin were from Vector laboratories. Gigapack packaging extracts was purchased from Stratagene. Forward or reverse primer and *EcoRI* methylase were from New England Biolabs. DNA polymerase I (Klenow fragment) was from United States Biochemical Corp., and DEAE membrane was from Schleicher and Schuell.

### Complement components

Human C3 was purified as described by Hammer *et al.* (1981). Factor I (Fearon & Austen, 1977) and Factor H (Nilsson *et al.*, 1975) were isolated from serum as previously described.

C3b was generated by incubating purified C3 with 1% (w/v) trypsin for 2 min at room temperature (Nilsson *et al.*, 1975), followed by separation on a Sephadex G-100 column (2.5 cm  $\times$  80 cm) equilibrated with saline (0.9% NaCl) buffered with 25 mM-sodium phosphate, pH 7.4 (PBS). iC3b was produced by incubating C3b (1 mg/ml) with Factor I (33  $\mu$ g/ml) and Factor H (170  $\mu$ g/ml) for 60 min at 37 °C. C3c and C3d were generated by incubating C3 with 2% trypsin for 60 min at 37 °C as described by Eggertsen *et al.* (1985).

C3 was labelled with <sup>125</sup>I by using Enzymobeads according to the manufacturer's recommendation (Nilsson & Nilsson, 1985). C3 retained haemolytic activity after the labelling procedure as determined by haemolytic titration using a C3-depleted serum (Nilsson & Nilsson, 1984).

### Preparation of erythrocytes coated with C3 fragments

EAC14<sup>oxy</sup>23b (EAC3b) and EAC14<sup>oxy</sup>23bi (EAC3bi) were prepared as previously described (Nilsson & Nilsson, 1985). The binding of C3 fragments to the cells was calculated by using <sup>125</sup>I-labelled native C3 (Nilsson & Nilsson, 1985).

### Antibody preparations

(a) **mAbs.** mAbs were raised in female BALB/c mice against SDS-denatured-reduced C3 as previously described (Nilsson *et al.*, 1987).

The mAbs were affinity-purified on Protein A-Sepharose. Culture supernatants containing mAbs were adjusted to 1.5 M-glycine/3 M-NaCl, pH 8.9, and were applied to a 4 ml Protein A-Sepharose column. After washing, the antibodies were eluted with 0.1 M-glycine, pH 2.8, and then dialysed against PBS. The antibodies were designated 4SD11.1, 4SD18.1, 7D9.2, 7D326.1 and 7D331.1.

(b) **Preparation of polyclonal anti-C3 antibodies.** Antisera against the intact  $\alpha$ -chain of human C3, isolated by gel chromatography, and against ovalbumin, were produced as previously described (Nilsson & Nilsson, 1982). Antibodies specific for the C3d fragment and 40 kDa C-terminal  $\alpha$ -chain portion of C3c were generated by immunizing rabbits with the corresponding fragments which had been separated by SDS/PAGE and eluted as previously described (Lambris *et al.*, 1984).

In order to diminish unspecific binding to *Escherichia coli*-derived proteins, all antisera were affinity-purified by chromatography on a C3- or ovalbumin-Sepharose column containing 5 mg of protein/ml of Sepharose. In the case of the anti-C3  $\alpha$ -chain antiserum, the bound C3 was denatured with SDS before purification. The columns were extensively washed with PBS and the bound proteins eluted with 0.2 M-glycine, pH 2.8. The eluates were dialysed against PBS.

The polyclonal-antibody preparation specific for the  $\alpha$ -chain of C3 and the mAbs were all of C3(D) specificity, i.e. the antibodies reacted with SDS-denatured C3 or C3 fragments, but not with native C3, as described by Nilsson & Nilsson (1982, 1985), and Nilsson *et al.* (1987) respectively.

### E.I.s.a. for detecting binding of anti-C3 antibodies to C3 fragments

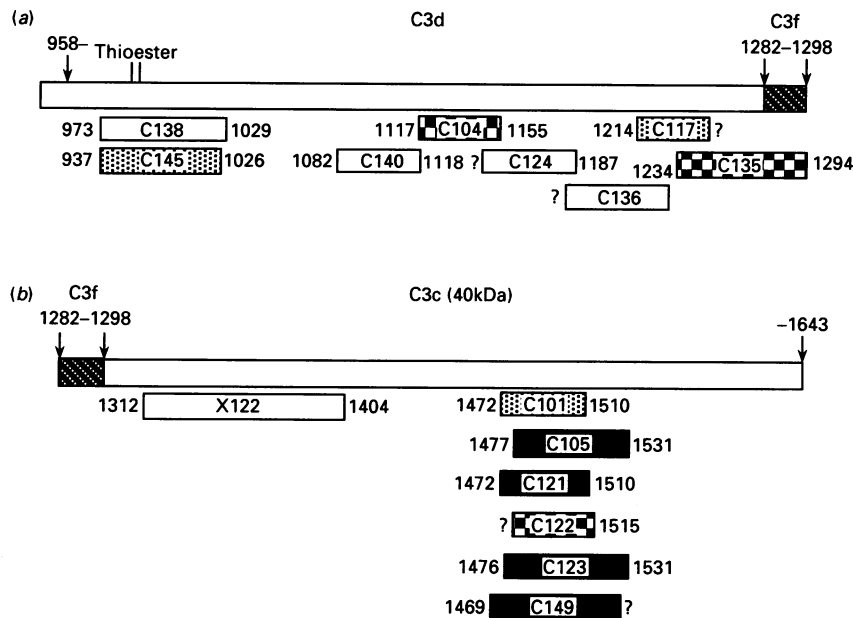
For the assessment of the specificity of mAbs and polyclonal antibodies to fluid-phase C3 fragments, an inhibition e.i.s.a. was used. Microtitre plates were coated with 200  $\mu$ l of C3 (3  $\mu$ g/ml) overnight at 4 °C and after that saturated with 1% (w/v) BSA. C3 deposited on polystyrene under these conditions adopts a highly denatured conformation, as shown by reactivity with antibodies raised against C3(D) epitopes (Elwing *et al.*, 1988).

A 50  $\mu$ l portion of serially diluted test antigen was preincubated with 50  $\mu$ l of a constant amount of monoclonal anti-C3 antibody (0.5–1  $\mu$ g/ml) for 60 min at room temperature in non-treated microtitre plates. After centrifugation, 100  $\mu$ l of supernatant was transferred to the coated microtitre plates and incubated for 60 min at room temperature. The binding of mAb and polyclonal antibody was detected by anti-mouse or anti-rabbit immunoglobulins conjugated with HRP.

### Generation and subcloning of C3 cDNA fragments

The pC3.11 cDNA clone of C3 was kindly provided by Dr. G. Fey (Scripps Clinic and Research Foundation, La Jolla, CA, U.S.A.) (de Bruijn & Fey, 1985). The insert was excised from the pAT153/*PvuII*/\* vector by digestion with *Clal* and *Sall*. This clone contains a *Sall* site, and the insert was thus obtained in two pieces. To obtain the region surrounding the *Sall* site intact, the clone was also digested in a separate reaction with *BstEII*. This releases a 2.3 kb fragment that spans the *Sall* site. This fragment was necessary so that the antigenic determinants in the region of the *Sall* site would not be lost in the expression library. The appropriate fragments were recovered from agarose gels by running the fragments into DEAE membrane. The fragments were eluted with buffered 1.5 M-NaCl and were ethanol-precipitated.

The expression library was made essentially as described by Nunberg *et al.* (1984). Each fragment was digested with DNAase I in 20 mM-Tris/HCl (pH 7.5)/1.5 mM-MgCl<sub>2</sub>/BSA (100  $\mu$ g/ml).



**Fig. 1. Graphical representation of the position of the expressed C3 fusion proteins in the  $\alpha$ -chain of C3 and their reactivity with polyclonal anti-(C3  $\alpha$ -chain) antibodies with C3(D) specificity**

The *N*-terminal and *C*-terminal amino acid residues of the expressed C3 fusion proteins are indicated, and the position of the C3 segment represented by the fusion protein is aligned with the  $\alpha$ -chain of C3. The reactivity of the polyclonal anti-C3 antibody is shown by means of various shadings: □, No binding; ▤, 1+; ▨, 2+; ■, 3+. The  $\alpha$ -chain segments shown are C3d and C3f (a) and C3f and the 40 kDa fragment of C3c (b).

Under these conditions, DNAase will make double-strand scissions (Crouse *et al.*, 1982). The digestion was calibrated thus that the mass average size of the digested fragments was about 200 bp. These fragments were electrophoresed through 1.5% agarose and the fragments between 200 and 300 bp were recovered by electroelution. An analytical gel of these fragments showed that there was still some (approx. 5%) fragments larger than 300 bp. The ends of these fragments were rendered blunt with the large fragment of DNA polymerase I in the presence of the four deoxynucleotides. [ $\alpha$ - $^{32}$ P]dCTP was added to lightly label the fragments and monitor them through subsequent steps. The fragments were methylated with *Eco*RI methylase and ligated to phosphorylated *Eco*RI linker 10-mers. Then they were digested with *Eco*RI and separated from excess linkers on Bio-Gel A15m. The fractions containing radioactivity were recovered by ethanol-precipitation, ligated to *Eco*RI-digested, dephosphorylated gt11 arms (Grossberger, 1987), and packaged in Gigapack packaging extracts. The packaging was amplified in the *E. coli* strain Y1088 (Vogelstein & Gillespie, 1979), and the amplified lysates were used for screening with either antibody (Grossberger *et al.*, 1989) or DNA probes (Benton & Davis, 1977).

#### Screening of the C3 cDNA minilibrary with anti-C3 antibodies

Screening was done directly on the library essentially as described by Grossberger *et al.* (1989). Affinity-purified polyclonal-antibody preparations against both the C3d fragment and the 40 kDa  $\alpha$ -chain subunit of C3c were used to screen the library initially.

After incubation with the primary antibody, the filters were incubated successively with biotin-coupled goat anti-rabbit Ig, with a complex of avidin/peroxidase-coupled biotin, and with the peroxidase substrate diaminobenzidine.

#### Generation and analysis of $\beta$ -galactosidase-C3 fusion proteins

Individual colonies of Y1089 recombinant lysogen were inoculated into 10 ml of Luria-Bertani medium (LB) and grown

for 3 h at 37 °C with good agitation. After the addition of 100 ml of 100 mM-isopropyl thio- $\beta$ -D-galactoside (IPTG), the culture was incubated overnight at 37 °C. The cells were harvested by centrifugation in a Beckman JA10 rotor at 5000 rev./min for 5 min at 30 °C. The cells were resuspended in 1 ml of 0.2 M-Tris/HCl (pH 7.5)/0.2 M-NaCl/1 mM-EDTA/5% (v/v) glycerol/1 mM-phenylmethanesulphonyl fluoride (PMSF)/1 mM-dithiothreitol (DTT) and frozen at -70 °C. After thawing the samples and addition of 1% SDS they were centrifuged in an Eppendorf centrifuge for 20 min. The supernatant was transferred to 12 ml conical glass tubes and 4 ml of ice-cold acetone was added to precipitate the proteins. After 2 h at -20 °C the proteins were pelleted by centrifugation at 5000 g, the pellet was resuspended in electrophoresis sample buffer, boiled, and an aliquot was analysed by SDS/PAGE. Duplicate gels were prepared. One was stained with Coomassie Blue to detect fusion-protein expression and the other was processed for immunoblotting. The electrophoresis was carried out in 12.5% gels on a Phast System apparatus (Pharmacia AB) according to the manufacturer's recommendations. The transfer to nitrocellulose membranes was achieved by passive diffusion at 70 °C for 30 min. After the transfer the nitrocellulose membrane was incubated in 1% (w/v) BSA in PBS for 30 min, followed by incubation for 60 min with the different anti-C3 antibodies. Bound polyclonal antibody was detected by biotinylated goat anti-rabbit Ig antibody followed by streptavidin-HRP conjugate, and bound anti-C3 mAb was detected by biotin-conjugated sheep anti-mouse Ig followed by streptavidin-biotin-HRP complexes.

#### cDNA sequencing

DNA from each clone was digested with *Mlu* (Grossberger, 1987), and the fragment containing the cloning site was recovered by binding to glass beads (Vogelstein & Gillespie, 1979). The fragments were denatured by boiling in the presence of either forward or reverse primer (New England Biolabs), and were

immediately chilled in a solid-CO<sub>2</sub>/ethanol bath (Manfioletti & Schneider, 1988). These primed templates were sequenced with Sequenase and [<sup>35</sup>S]dATP. The distal boundary of each was determined by sequencing through the entire insert.

## RESULTS

### Production of $\beta$ -galactosidase-C3 fusion proteins

The cDNA clone which spans oligonucleotide 708–5049, corresponding to amino acid residues 177–1663 of C3 (clone pC3.11) was used to construct an expression library. Short DNAase I-generated cDNA fragments encoding random portions of C3 were cloned into the *Eco*RI site of the  $\beta$ -galactosidase gene of the vector  $\lambda$ gt 11. The library was screened with polyclonal antibody preparations against both the C3d fragment and the 40 kDa  $\alpha$ -chain subunit of C3c. The boundaries of the clones reacting with the anti-C3 antibodies screening positive clones were determined by sequencing them from both ends. A total of 15 different fusion proteins recognized by the antibodies were produced in the *E. coli* strain Y1088. These proteins represent a library of  $\beta$ -galactosidase-linked C3 peptides

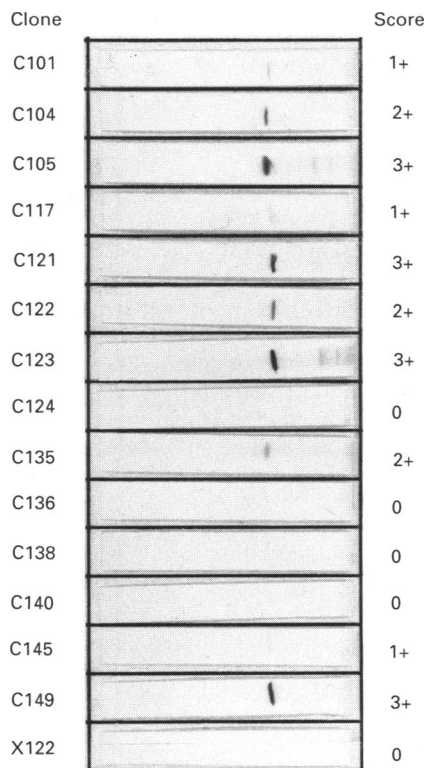


Fig. 2. Binding of polyclonal anti-C3(D) antibodies to different  $\beta$ -galactosidase-C3 fusion proteins

C3 was conjugated to CNBr-activated Sepharose (5 mg of protein/ml of gel). Rabbit antiserum to the  $\alpha$ -chain of C3 [C3(D) specificity] were passed through the Sepharose column and the bound antibodies were eluted with 0.2 M-glycine, pH 2.8. A total of 15 different fusion C3 proteins were subjected to SDS/12.5% PAGE and then transferred to nitrocellulose membranes. After saturation of the membranes in 1% BSA for 30 min, they were incubated for 60 min with the polyclonal anti-C3 antibody preparation in PBS containing 0.1% BSA and 0.1% Tween 20. After rinsing the membranes, the bound anti-C3 antibodies were detected by anti-rabbit immunoglobulin conjugated with HRP. The binding of the anti-C3 antibodies is shown, and the arbitrary binding, based on intensity (0–3), was estimated and presented for each membrane strip.

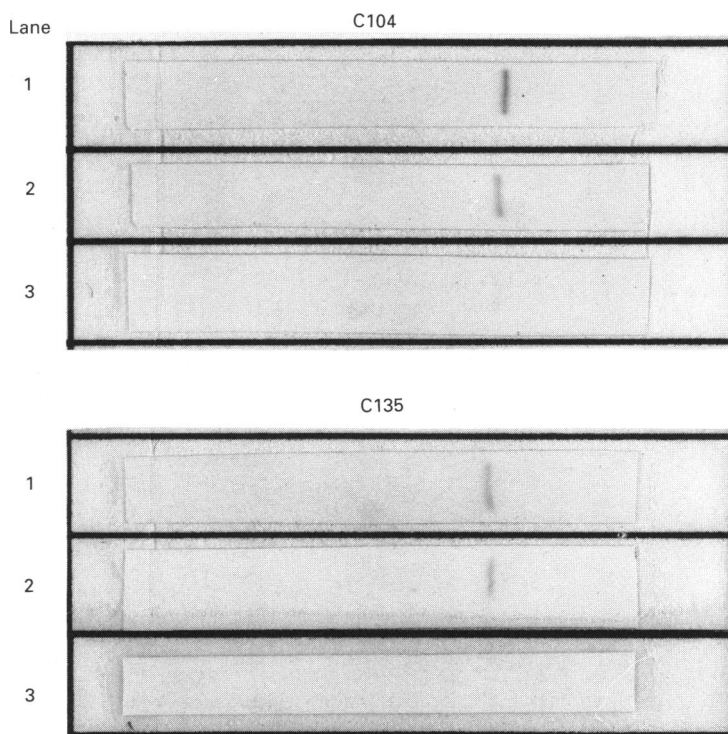


Fig. 3. Reactivity of polyclonal anti-C3(D) antibodies towards surface and fluid-phase C3 fragments

The specificity of the polyclonal anti-C3(D) antibodies, reacting with the fusion proteins, for surface-bound or fluid-phase C3 fragments was analysed by incubating them with soluble or bound C3b/iC3b before they were analysed by Western blotting. Lane 1, untreated antibodies; lane 2, preincubated with a mixture of equal amounts of C3b and iC3b; lane 3, preincubated with a mixture of equal amounts EAC3b and EAC3bi. Only the binding to c104 and c135 was affected by preincubation with C3 fragments.

with known amino acid sequence. Their delineation in the  $\alpha$ -chain of C3 is depicted in Fig. 1.

### Reactivity of the affinity-purified rabbit anti-C3 antibodies with C3 fragments and fusion proteins

Affinity-purified rabbit anti-C3(D) antibodies (specific for  $\alpha$ -chain) were tested for binding to the fusion proteins. The *E. coli* extracts were subjected to SDS/PAGE, followed by Western-blot analysis employing the polyclonal antibody. In Fig. 2 the binding of the affinity purified anti-C3 antibodies is shown, and the binding reactivity of the anti-C3 antibodies to each clone was scored based on intensity. The anti-ovalbumin antibodies did not bind to any of the C3 fusion proteins (results not shown).

In Fig. 1 the score of the binding for each protein is graphically presented and shows that the strongest binding was to proteins corresponding to the C-terminal half of the 40 kDa subunit of C3c, i.e., proteins C105 (3+), C121 (3+), C122 (2+) and C123 (3+). In contrast, no binding was observed to the protein X122. The antibodies did also bind to different clones in the C3d domain, i.e. C145 (1+), C104 (2+), C117 (1+) and C135 (2+).

To establish whether the affinity-purified anti-C3 antibodies bound to epitopes that are expressed by bound or soluble C3 fragments, the antibody preparation was preincubated with mixtures of equal amounts of soluble C3b and iC3b or EAC3b and EAC3bi. In Fig. 3 the binding of the different anti-C3 antibodies to proteins C104 and C135 is shown. Identical binding strength was demonstrated to both proteins with non-adsorbed antibody and with antibody adsorbed with soluble C3b plus

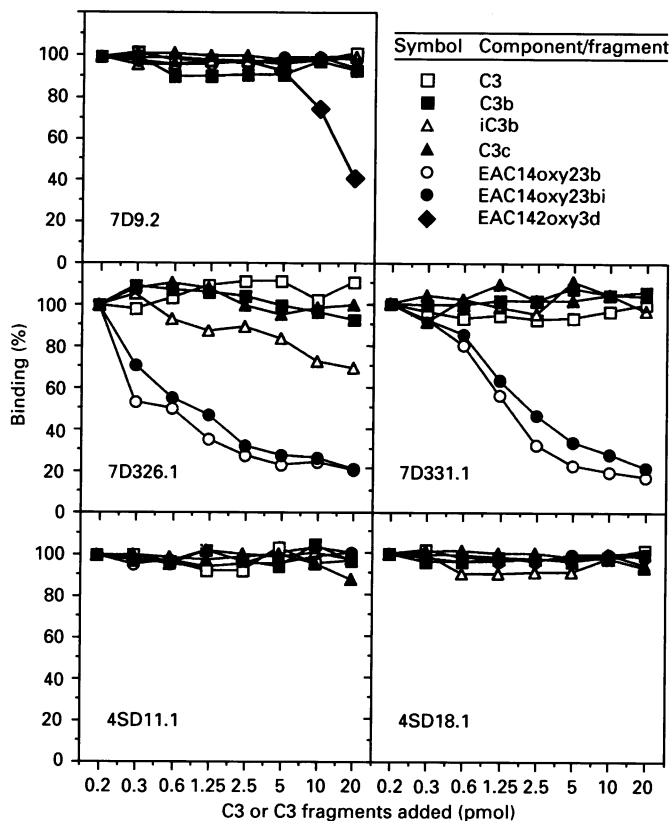


Fig. 4. Reactivity of monoclonal anti-C3(D) antibodies for surface-bound and fluid-phase C3 fragments

Microtitre plates were coated with 200  $\mu$ l of C3 (3  $\mu$ g/ml) overnight at 4 °C and thereafter saturated with 1% (w/v) BSA. A 50  $\mu$ l portion of serially diluted test antigen was preincubated with 50  $\mu$ l of a constant amount of 4SD11.1, 4SD18.1, 7D9.2, 7D326.1 or 7D331.1 (0.5–1  $\mu$ g/ml) for 60 min at room temperature in non-coated microtitre plates. After centrifugation, 100  $\mu$ l of supernatant was transferred to the C3-coated microtitre plates and incubated for 60 min at room temperature. The binding of mAb was detected by anti-mouse Ig conjugated with HRP. The binding of the monoclonal antibodies in the absence of test antigen (expressed as  $A_{492}$ ) ranged between 0.9 and 1.1.

iC3b. In contrast, no binding was shown with the antibody preparation adsorbed with EAC3b plus EAC3bi. The binding to other fusion proteins was not diminished by absorption of the antibody with any of the C3 fragments.

#### Reactivity of monoclonal anti-C3 antibodies with C3 fragments and fusion proteins

Monoclonal anti-C3(D) antibodies were tested for binding to fluid-phase and erythrocyte-bound C3 fragments by an inhibition e.i.s.a. Fig. 4 shows that mAb 7D326.1 and 7D331.1 were inhibited by EAC3b and EAC3bi. MAb 7D326.1 was also inhibited by fluid-phase iC3b, but to get a similar inhibition to that of EAC3bi, a 50-fold higher dose of fluid-phase iC3b was required. 7D9.2 was inhibited exclusively by EAC3d cells. Unlike these three antibodies, 4SD11.1 and 4SD18.1 were not inhibited by any of the C3 fragments used.

MABs 4SD11.1, 4SD18.1, 7D9.2, 7D326.1 and 7D331.1 were tested for binding to the expressed fusion proteins. The fusion proteins were SDS/PAGE followed by Western-blot analysis. As Fig. 5 shows, 7D9.2 reacted exclusively with the fusion protein C140, but not with any other protein. Similarly 7D326.1 and 7D331.1 bound specifically to fusion protein X122. Unlike the

other antibodies, 4SD11.1 and 4SD18.1 bound to several clones, i.e., clones C105, C121 and C123. Yet their reactivity differed in that 4SD11.1 bound strongest to C121 and 4SD18.1 to C105. The binding of mAbs to the expressed C3 proteins is summarized in Table 1.

#### DISCUSSION

The conformational changes in the molecule that accompany the proteolytic cleavage events during activation/inactivation of C3 and C3 fragments have been detected by using an assortment of chemical probes and spectral and solution scattering techniques (Molenaar *et al.*, 1975; Isenman & Cooper, 1981; Pangburn *et al.*, 1981; Isenman, 1983; Perkins & Sim, 1986). Although these techniques allowed the analysis of fluid-phase C3 fragments, technical difficulties have hampered a similar analysis of target surface-bound fragments. However, the use of mAbs has overcome these difficulties, and, in fact, been shown to be more sensitive in probing conformational changes in proteins than the low-resolution spectroscopic methods (Collawn *et al.*, 1988). By identifying the segment of the amino acid sequence that contains the antibody epitope, parts of the polypeptide chain involved in the conformational changes, or in forming surface structures of the molecule, have been recognized. Such studies of fluid-phase and bound C3 fragments have been performed in our and other laboratories using mAbs specific for neoantigenic epitopes in conjunction with overlapping synthetic peptides (Lambris *et al.*, 1985; Burger *et al.*, 1988; Alsenz *et al.*, 1990; Nilsson *et al.*, 1990). These studies have identified regions in the C3a, iC3b, C3dg and C3d fragments of C3 which are associated with conformational changes that occur upon degradation of the C3 molecule.

Evidence that the conformation of C3 differs in the soluble, as compared with the bound, forms of C3 fragments is that C3(D) epitopes are exposed under these conditions (Nilsson & Nilsson, 1982; Nilsson *et al.*, 1987, 1989). This is based on the observation that, during denaturation by SDS, exposure of C3(D) antigens by C3 or C3b is very well correlated to a profound conformational change detected by c.d. in the far-u.v. spectrum in these molecules. Under physiological conditions, C3(D) epitopes are expressed preferentially by bound C3 fragments and only to a small extent by the corresponding soluble molecule. By using mAbs and polyclonal antibodies specific for C3(D) epitopes for studies of the folding of the polypeptide chains of the bound C3 molecule, regions in the molecule involved in conformational changes associated with binding of C3 fragments to a target are identified.

In the present study both mAbs and polyclonal anti-C3 antibodies with C3(D) specificity, were used to localize epitopes on C3 specifically expressed by surface-bound and/or denatured fragments. These epitopes were mapped by using C3 fragments, expressed as  $\beta$ -galactosidase fusion proteins, representing overlapping segments of the C3d fragment and the 40 kDa subunit of C3c. To distinguish between epitopes that were expressed by surface-bound and/or denatured C3 fragments, the antibodies were adsorbed with fluid-phase or erythrocyte-bound C3b/iC3b. The binding of the polyclonal anti-C3 antibodies ( $\alpha$ -chain-specific) to fusion proteins C104 and C135 was inhibited by EAC3b/iC3b, but not with the corresponding fluid-phase C3 fragments. This indicated that the segments of C3 spanning residues 1117–1155 and 1234–1294 accommodate epitopes expressed specifically by both erythrocyte-bound C3b and iC3b fragments (Table 1). Similarly, the binding of mAbs 7D326.1 and 7D331.1 to fusion protein X122 (spanning residues 1312–1404 of C3) was inhibited by EAC3b/iC3b and that of mAb 7D9.2 to C140 (residues 1312–1404) by EAC3d. None of the mAbs were inhibited by the corresponding fluid-phase C3 fragments. The

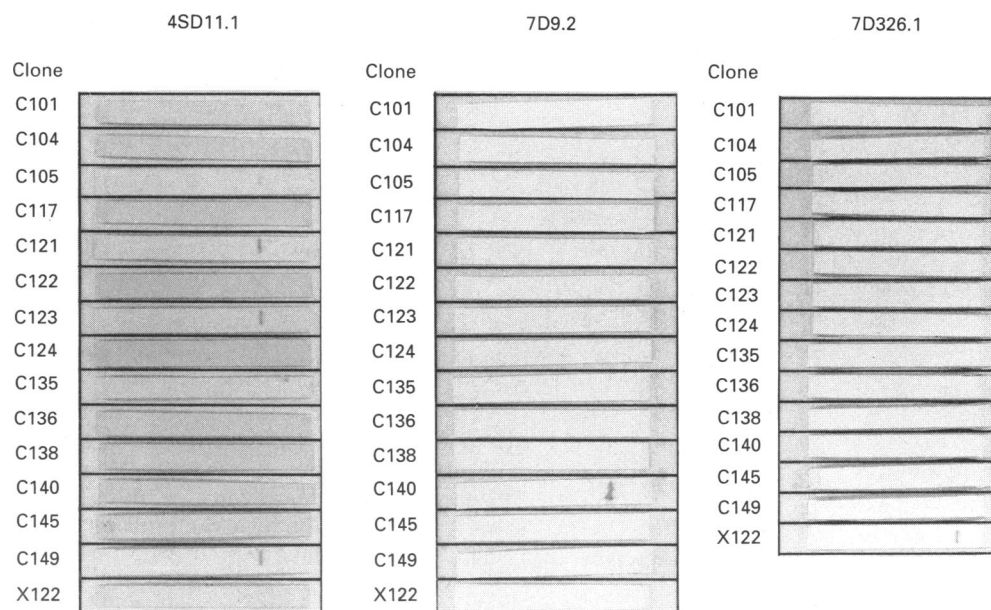


Fig. 5. Binding of monoclonal anti-C3(D) antibodies to  $\beta$ -galactosidase-C3 fusion proteins

Reduced  $\beta$ -galactosidase-C3 fusion proteins were run in SDS/PAGE (12.5%) and transferred to nitrocellulose membranes. After saturation of the membranes in 1% BSA for 30 min, the membranes were incubated, for 60 min, with 1  $\mu$ g of 4SD11.1, 7D236.1 or 7D9.2, in PBS containing 0.1% BSA and 0.1% Tween 20. After washing the membranes the bound antibody was detected by anti-mouse Ig followed by streptavidin-biotinylated HRP complex.

Table 1. Summary of the anti-C3 mAb reactivity with C3 fusion proteins

mAb	Recognized C3 fragments	cDNA clone	C3 residues
7D9.2	C3d*, EAC3d†	C140	1082-1118
7D326.1	C3c*, EAC3b,† EAC3bi†	X122	1312-1404
7D331.1	C3c*, EAC3b, EAC3bi†	X122	1312-1404
4SD11.1	C3c*	C105 C121‡ C123 C149	1477-1531 1472-1510 1476-1531 1469-?
4SD18.1	C3c*	C105‡ C121 C123 C149	1477-1531 1472-1510 1476-1531 1469-?

\* Western-blot analysis.

† E.l.i.s.a. as described in Fig. 4.

‡ Clone to which mAb preferentially binds.

remainder of the mAbs that recognized fusion proteins were not inhibited by any type of C3 fragment, indicating that the antibodies bound to epitopes that are hidden in physiological C3 fragments but expressed by denatured molecules.

This study focuses on C3(D) epitopes located in the  $\alpha$ -chain of C3. Combined with previous studies performed in our laboratories (Alsenz *et al.*, 1990; Nilsson *et al.*, 1990), C3(D) epitopes have been found to be distributed throughout the whole  $\alpha$ -chain (Fig. 6). As shown herein, epitopes which are exposed exclusively by denatured C3 or C3 fragments have been found in the C-terminal end of the polypeptide chain. Previous studies have demonstrated that a similar set of epitopes are found in the N-terminal end between amino acid residues 741 and 758 (Alsenz *et al.*, 1990). By contrast, C3(D) epitopes expressed both by physiologically bound and denatured C3 fragments are found

more in the centre of the primary sequence of the  $\alpha$ -chain. In addition to those found in this study within the segments 1082-1155, 1234-1294 and 1312-1414, such epitopes were found between Arg<sup>929</sup> and Ser<sup>946</sup> using mAbs (7D264.6, 7D84.1) and synthetic peptides (Nilsson *et al.*, 1990). The association between the location of the epitopes and the different functional sites in C3 is still unclear and requires further investigation.

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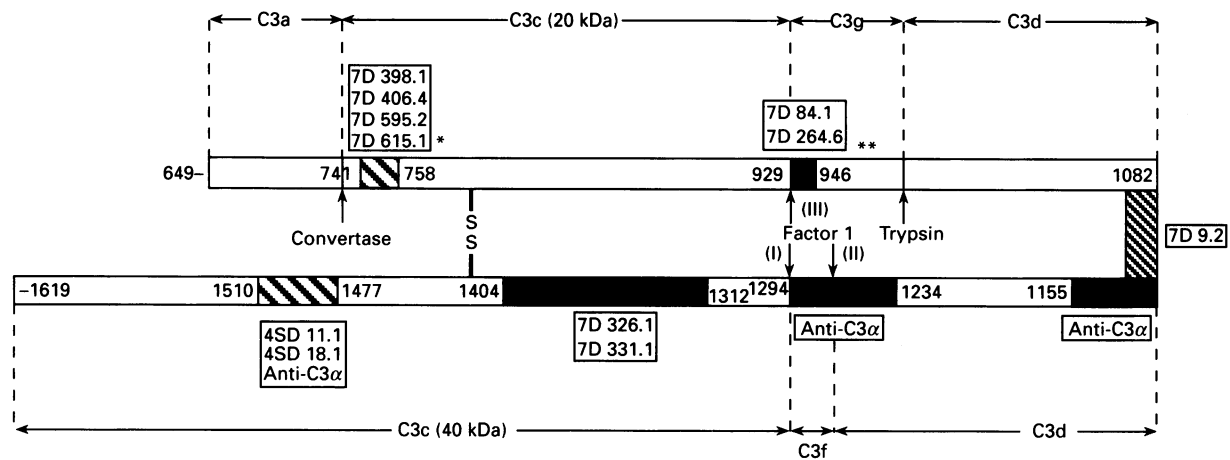


Fig. 6. Localization of C3(D) epitopes detected by mAbs and polyclonal antibodies in the  $\alpha$ -chain of C3

Previously published C3(D) epitope locations between amino acid residues 929 and 946 (\*\*; Nilsson *et al.*, 1990) and between 741 and 758 (\*; Alsenz *et al.*, 1990) are included in the Figure. Key to shadings: ▨, exposed on microtitre plate-fixed C3; ▩, exposed on EAC3b-3bi and on microtitre plate-fixed C3; ▪, exposed on EAC3d and on microtitre plate-fixed C3.

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