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Serine 132 Is the C3 Covalent Attachment Point on the CH1 Domain of Human IgG1*

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Luis Vidarte‡, Carlos Pastor‡, Sebastian Mas‡, Ana Belen Blázquez‡, Vivian de los Rios‡§, Rosa Guerrero‡, and Fernando Vivanco‡¶

From the ‡Department of Immunology, Fundación Jímenez Díaz, 28040 Madrid, Spain and §Department of Biochemistry and Molecular Biology I, Universidad Complutense, 28040 Madrid, Spain

The covalent binding of C3 (complement component C3) to antigen-antibody complexes (Ag·Ab; immune complexes (ICs)) is a key event in the uptake, transport, presentation, and elimination of Ag in the form of Ag·Ab·C3b (IC·C3b). Upon interaction of C3 with IgG·IC, C3b·C3b·IgG covalent complexes are formed that are detected on SDS-polyacrylamide gel electrophoresis by two bands corresponding to C3b·C3b (band A) and C3b·IgG (band B) covalent complexes. This allows one to evaluate the covalent binding of C3b to IgG antibodies. It has been described that C3b can attach to both the Fab (on the CH1 domain) and the Fc regions of IgG. Here the covalent interaction of C3b to the CH1 domain, a region previously described spanning residues 125–147, has been studied. This region of the CH1 domain is exposed to solvent and contains a cluster of six potential acceptor sites for ester bond formation with C3b (four Ser and two Thr). A set of 10 mutant Abs were generated with the putative acceptor residues substituted by Ala, and we studied their covalent interaction with C3b. Single (Ser-131, Ser-132, Ser-134, Thr-135, Ser-136, and Thr-139), double (positions 131–132), and multiple (positions 134–135-136, 131–132-134–135-136, and 131–132-134–135-136–139) mutants were produced. None of the mutants (single, double, or multiple) abolished completely the ability of IgG to bind C3b, indicating the presence of C3b binding regions other than in the CH1 domain. However, all mutant Abs, in which serine at position 132 was replaced by Ala, showed a significant decrease in the ability to form C3b·IgG covalent complexes, whereas the remaining mutants had normal activity. In addition we examined ICs using the $F(ab')_2$ fragment of the mutant Abs, and only those containing Ala at position 132 (instead of Ser) failed to bind C3b. Thus Ser-132 is the binding site for C3b on the CH1 domain of the heavy chain, in the Fab region of human IgG.

On exposure to most antigens a host responds by synthesizing specific antibodies that subsequently interact with the inciting antigens, forming antigen-antibody complexes (immune complexes (ICs)[1]). In fluids containing complement, the process of IC formation and the attachment of complement components occur simultaneously. Thus, the formation of Ag·Ab-complement complexes is a normal way of eliminating most soluble antigens from a host (1–3). In this process the covalent incorporation of C3b into the IC lattice is the critical event that conditions the fate of the IC·C3b (2–4). Upon interaction of serum C3 with IgG·IC, $C3b_2$·IC covalent complexes are immediately formed. In this macromolecular structure C3b molecules are covalently bound to each other and to the IgG, preferentially through ester bonds (4, 5). These C3b·C3b dimers are subsequently converted into iC3b·iC3b·IgG complexes by the complement regulators (5, 6), which are detected on SDS-PAGE by two bands of molecular composition C3α65-C3α43 (band A) and C3α65-heavy chain of the antibody (band B), which correspond to C3b·C3b and C3b·IgG covalent interaction, respectively (5, 7, 8). The size of the two proteins involved (150 kDa and about 180 kDa for IgG and C3b, respectively) and the lability of the ester linkage have made it difficult to identify the amino acids involved. Exact knowledge of the site(s) of attachment has become more important with the development of genetically engineered antibodies and chimeras with therapeutic applications (9).

Two main areas for C3b binding have been described on the IgG, one located in the CH1 domain of Fab (10, 11) and the other in the Fc region (8). The binding site on the CH1 domain comprises the first loop and part of the adjacent β strands (residues 125–147; Eu numbering) (33). This region is exposed to solvent and contains eight possible acceptor residues (four Ser, two Thr, and two Lys). Lys residues are not exposed to solvent and not expected to be potential sites for amide bond formation with C3. Using synthetic peptides corresponding to this region of IgG1, Sahu and Pangburn (12) suggest that Thr-135 would be the preferred site for C3b binding. A more direct approach to identifying the site(s) of C3b attachment is by site-directed mutagenesis of this region of IgG. Thus, in this work we have generated a set of chimeric mutant antibodies in which one or more hydroxylated amino acid residues were substituted by Ala. We studied their covalent interaction with C3b. Data indicate that Ser-132 is the acceptor residue for the covalent binding of C3b on the CH1 domain of human IgG1.

EXPERIMENTAL PROCEDURES

Chimeric Mutant Antibodies—A chimeric antibody was constructed with murine V regions (VH and VL) of a monoclonal antibody specific for human serum albumin (HSA) (HSA-4; clone LGF1/4.3.1.B9) and human constant regions (Cγ1 and Cκ) as previously described (7, 13). Mutant antibody genes were initially constructed in pBlueScript II SK (Stratagene), and this vector was used as a template for site-directed

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¶ To whom correspondence should be addressed: Dept. of Immunology, Fundación Jímenez Díaz, Avda. Reyes Católicos 2, 28040 Madrid, Spain. Tel.: 34915498446; Fax: 34915448246; E-mail: fvivanco@fjd.es.

[1] The abbreviations used are: IC, immune complex or antigen-antibody complex; Ag, antigen; Ab, antibody; C3, the third component of the Complement system; C3α65 and C3α43, degradation fragments of C3α chain; H and L, heavy and light chains of IgG; scAb, single chain Ab, a recombinant Ab without CH1 domain; chAb, chimeric antibody; Fd, N-terminal half of H chains (VH and CH1 domains); HSA, human serum albumin; PAGE, polyacrylamide gel electrophoresis.

mutagenesis using polymerase chain reaction (ExSite kit, Stratagene). Mutations were generated by changing hydroxylated residues by Ala. The complete H and L genes were sequenced for every Ab confirming the presence of the mutations. The non-Ab-producing myeloma cell line Sp2/0 was used for expression of the complete Abs using the pING2003E vector as described (7, 8, 13).

Purification of Antibodies and F(ab′)$_2$ Fragments—Antibodies (monoclonal HSA4, chimeric and chimeric-mutated, single-chain antibody (scAb) see below C3 binding assays) were purified from ascitic fluid by affinity chromatography. They were precipitated from ascites by ammonium sulfate and purified on protein-A Sepharose (Amersham Pharmacia Biotech). IgG was eluted with citrate buffer, pH 3.5, neutralized, and loaded onto an HSA-Sepharose column, which separates the chimeric antibodies (HSA-specific) from mouse host IgGs (7, 8). F(ab′)$_2$ fragments were obtained by pepsin digestion of the corresponding IgG in acetate buffer, pH 4.6 (14), followed by chromatography on a Superdex 75 column (fast protein liquid chromatography) and affinity chromatography on protein A-Sepharose as described above (7, 8). Purity of antibodies and F(ab′)$_2$ fragments was verified by SDS-PAGE under reduced and non-reduced conditions and visualized by staining with Coomassie Blue R-250 (Sigma).

Biosensor Analysis of Antigen Binding—Ag·Ab binding interactions were monitored by surface plasmon resonance on an IAsys instrument (Fissons Applied Sensor Technology). HSA was immobilized into the cuvette precoated with a high molecular weight carboxymethylated dextran according to the conditions recommended by the manufacturer. For determination of K_d values, binding was examined using four to six different antibody concentrations in the 50–200 nM range in phosphate-buffered saline (0.02 M Na_3PO_4, 0.15 M NaCl, pH 7.2). Kinetic analysis was performed using the FASTfit software provided by the manufacturer, which yielded the values for K_{on} and K_{diss} at each antibody concentration. An ϵ (0.1%, 1 cm, 280 nm) of 1.3 was used for all the antibodies.

SDS-PAGE, Fluorography, and Blotting—SDS-PAGE and two-dimensional gel electrophoresis was carried out as previously described (5, 7). Fluorography of gels containing ^{14}C-labeled samples and quantitation of radioactive bands were performed according to Laskey (15) using Kodak X-Omat-S film as described (16). Except for the use of nitrocellulose membranes, blotting experiments were carried out as described (5, 7). Human C3, used as an immunogen, was isolated and purified by fast protein liquid chromatography (17). Polyclonal anti-human C3 and anti-Fc were prepared in rabbits as previously described (17). The human Fc fragment was obtained by papain digestion of IgG and isolated as described (18). A peroxidase-conjugated secondary antibody (anti-human Fc$_\gamma$; Nordic, The Netherlands) with the ECL system was used. In some cases, the same membrane was sequentially (after striping) used with both antisera to show the presence of C3 and the Ab in the same band.

Antigen-Antibody Aggregates—HSA-antibody aggregates were formed at equivalence as described previously in detail (7, 13, 19). The precipitin curves were virtually identical for all the Abs used (monoclonal HSA4, chimeric and mutants).

C3 Binding Assays—These assays were performed with the monoclonal HSA4, chimeric antibody, a scAb bearing the same V regions but devoid of CH1-CL module (7) and the chimeric mutant Abs and their corresponding F(ab′)$_2$ fragments. HSA-IgG (or F(ab′)$_2$) aggregates (100 μg) were washed twice with 50 mM Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl and incubated with 800 μl of normal human serum under conditions in which only the alternative pathway of complement is activated (7, 8, 13, 19). After the incubation period, the reaction was stopped with 1 ml of ice-cold phosphate-buffered saline, and the precipitates were washed three times with the same buffer and resuspended in sample buffer for SDS-PAGE.

To detect the binding of C3b to the aggregates, 6.25 μg of iodo-[1-^{14}C]acetamide (2.22 Gbq/mmol; Amersham Pharmacia Biotech) were included in the normal human serum. This labeled the covalent complexes in which C3b (which is radioalkylated in the nascent SH group) was been incorporated (5, 7, 8, 19). For comparison, ICs formed with the chimeric antibody (chAb) and with the scAb were included as positive and negative controls, respectively, in each set of assays. ICs incubated with normal human serum-EDTA, which prevents the classical as well as the alternative pathways of complement, were included as the control.

Band B corresponds to C3α65-H covalent adducts that can be used as a measure of the capacity of the Ab to covalently bind C3 (5, 8, 11). Hence, the C3b covalent binding ability of the different mutant Abs was evaluated, quantitating band B in the autoradiographies or Western blots, using a Molecular Dynamics laser densitometer (Image Quant 3.1 software) and normalized to the sum of the intensity of bands B and H (the total amount of H chain). For each chimeric or mutant Ab, a minimum of three independent binding assays was performed, and the average value and S.D. was determined.

Antibody	K_a (M^{-1})	SEQUENCE (EU Numbering)														
		128			131	132			134	135	136			139		142
Chimeric	1.26 e-007	L	A	P	**S**	**S**	K	**S**	**T**	**S**	G	G	**T**	A	A	L
S131A	1.09 e-007	L	A	P	A	**S**	K	**S**	**T**	**S**	G	G	**T**	A	A	L
S132A	1.38 e-007	L	A	P	**S**	A	K	**S**	**T**	**S**	G	G	**T**	A	A	L
SS12	1.25 e-007	L	A	P	A	A	K	**S**	**T**	**S**	G	G	**T**	A	A	L
S134A	1.12 e-007	L	A	P	**S**	**S**	K	A	**T**	**S**	G	G	**T**	A	A	L
T135A	1.23 e-007	L	A	P	**S**	**S**	K	**S**	A	**S**	G	G	**T**	A	A	L
S136A	1.35 e-007	L	A	P	**S**	**S**	K	**S**	**T**	A	G	G	**T**	A	A	L
STS456	1.51 e-007	L	A	P	**S**	**S**	K	A	A	A	G	G	**T**	A	A	L
SS12STS456	1.44 e-007	L	A	P	A	A	K	A	A	A	G	G	**T**	A	A	L
T139A	1.31 e-007	L	A	P	**S**	**S**	K	**S**	**T**	**S**	G	G	A	A	A	L
SS12STS456T9	1.16 e-007	L	A	P	A	A	K	A	A	A	G	G	A	A	A	L

FIG. 1. **Affinity constants of the chimeric and mutant Abs.** The sequence of the CH1 domain-spanning residues 128–142 is shown. Residues in *bold* indicate the possible C3b binding sites. *Underlined* positions indicate mutations. *Bold vertical lines* separate the first loop of the CH1 domain (residues 132–139) from the residues belonging to the adjacent β strands.

Modeling the C3b Binding Region of Mutant Antibodies—To study the possible effects that the mutations could produce on the conformation of the loop (positions 128–142), we modeled this region for the mutant Abs using as a reference three-dimensional structures of antibodies of the same isotype (γ,κ) that had been resolved at less than 2.9 Å, taken from the Protein Data Bank (Brookhaven) (20–22), and visualized them with Rasmol v.2.6 or Swiss Pdb-Viewer v.3.1. The alignment of sequences and changes in amino acid side chains were carried out using the Swiss-Pdb Viewer program. Data were submitted to the automated protein-modeling server (Swiss model; GlaxoSmithKline, Geneva; www.expasy.ch) for minimum energy calculations (Gromos program) (23, 24).

RESULTS

Characterization of the Chimeric and Mutant Antibodies—The method used to purify Abs prevents the presence of Ab molecules from the host, interfering with the C3 binding assays and masking the results. All the Abs exhibited K_a constants ranging from 1.1 to 1.5 $\times$ 10^{-7} M (Fig. 1; chimeric Ab, 1.26 $\times$ 10^{-7} M), indicating that the mutations introduced in the CH1 domain do not affect the interaction with the Ag. Furthermore, the precipitin curves were virtually identical for all the mutants. This was confirmed using ^{125}I-Abs and measuring the cpm in the pellet and supernatant (not shown). Fig. 2 shows the structure of CH1 domain and the position of the loop involved in the C3b binding. The production of chimeric or mutant Abs by SP2/0 cells was low, around 0.5–0.8 mg/ml ascites. However, because the production of recombinant Abs structurally identical to the native Abs was an essential requirement and dependent on glycosylation, we selected these cells for the production of the Abs. The purification process, followed by SDS-PAGE, is shown in Fig. 3. It can be observed by comparing *lanes 3* and *4* that the polyclonal host Abs are removed onto the HSA-Sepharose column and the chimeric Abs appear with the characteristic single monoclonal bands defining the H and L chains at about 50 and 25 kDa. All the Ab mutants and the scAb (7) were affinity-purified in the same way and used in the subsequent experiments.

C3 Covalent Binding to Chimeric Antibody—To evaluate the ability of the mutant Abs to bind C3b, it was essential to determine the C3b binding capacity of the chAb used as reference and to check whether this Ab binds covalently with C3b forming C3b·C3b covalent adducts (1). Fig. 4 shows the SDS-PAGE analysis of this C3b binding assay carried out in the presence of [1-^{14}C]acetamide. When C3b binds to rabbit or human IgG1 immune complexes, covalent adducts are formed that are detected by SDS-PAGE as two characteristic bands (named A and B, see the Introduction).

Fig. 4*A* shows the presence and identification of these bands with ICs formed with the chAb (stained gel). Autoradiography of this gel shows that both bands, A and B, appear intensively

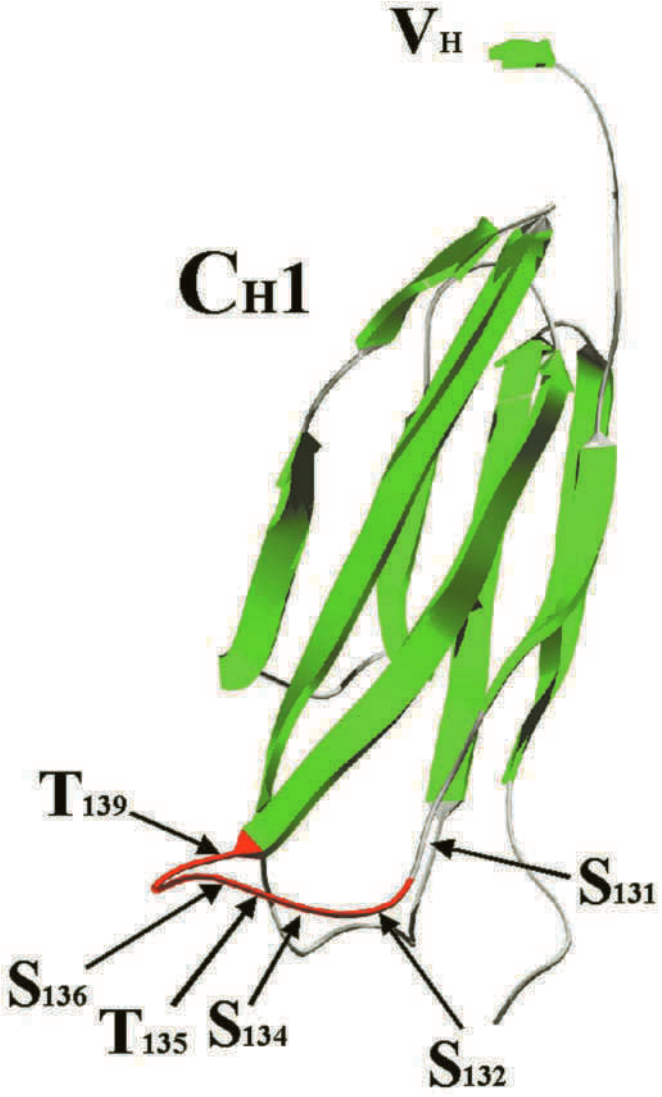

FIG. 2. **Shown is a three-dimensional structure of the CH1 domain showing the β-strands (*green ribbons*) and the loop involved in C3b binding (residues 132–139) in *red*.** The position of the six hydroxylated residues, which were mutated to Ala, are marked. V_H, variable domain of H chain.

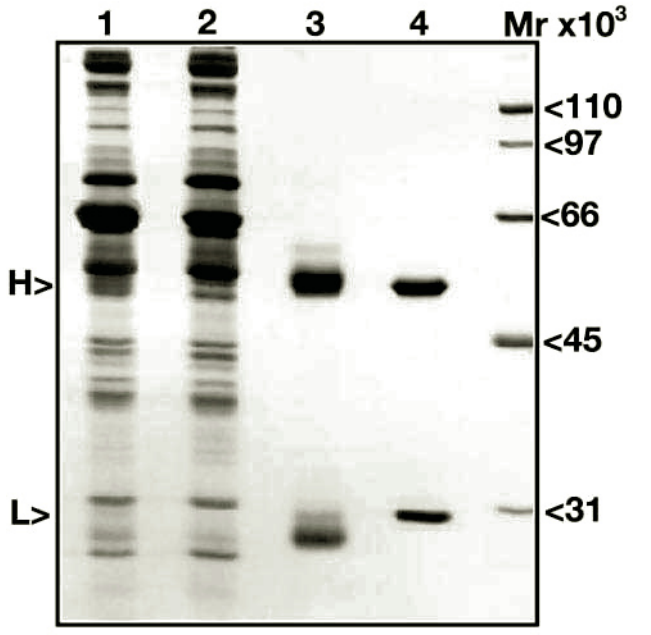

FIG. 3. **Chimeric antibody was purified from ascitic fluid (*lane 1*) by selective precipitation with ammonium sulfate 2M (*lane 2*) followed by two steps of affinity chromatography in protein A-Sepharose (*lane 3*) and antigen (*HSA*) coupled to Sepharose (*lane 4*).**

labeled, indicating the presence of C3b in both bands. When EDTA was included in the assay, which prevents activation of complement, the high molecular mass bands are not formed, indicating the specificity of the assay. To determine the composition of these bands, they were treated with anti-Fc and anti-C3 antisera in Western blot experiments. Band A was only positive with the anti-C3 antiserum, whereas band B was stained with both antisera, indicating the presence of C3 and H chain of the Ab in this band (Fig. 4*A*). Taken together these data demonstrate the presence of C3 fragments (bands A and B) and H (band B) in the high molecular mass bands.

Molecular Composition of Chimeric Ab·C3b Covalent Complexes—The nature of the polypeptides present in each A and B band was determined by two-dimensional electrophoresis. A track as shown in the first dimension gel (Fig. 4*A*) but using 500 μg of IC was treated with 1 M NH_2OH for 90 min at 37 °C and applied in the second gel. The result is presented in Fig. 4*B* (stained gel). Band A of the first dimension was mostly dissociated into spots of 65 and 43 kDa, indicating that it contains C3α65·C3α43 covalent adducts linked by an ester bond sensitive to hydroxylamine. A residual amount of band A does not dissociate, remaining in the diagonal. Likewise, band B released two major spots of 65 and 50 kDa, which correspond to C3α65·H covalent complexes sensitive to hydroxylamine. The same spot pattern was obtained if the ICs were previously alkylated with iodoacetamide. These data indicate that chimeric antibody, like rabbit or human IgG1, activates the alternative pathway of complement, forming C3b·C3b·chAb covalent complexes (1, 5, 7).

C3b Covalent Binding to Mutant Antibodies—The first mutant antibodies to be examined were those with multiple substitutions (Ser-134–Thr-135–Ser-136: mutant STS456) in the central positions of the loop. Fig. 5*A* shows the results of these assays in comparison with the chimeric antibody (positive control) and to scAb, which lacks the CH1 domain (negative control). The amount of C3b bound by scAb is significantly lower than that of a complete antibody since it binds C3b exclusively on the Fc region (7). It is observed that mutant STS456 was not affected by the multiple mutations at three consecutive positions (134–136), and the intensity of band B was comparable with that chimeric antibody. Modeling of these mutated positions showed small rearrangements that involved minimum displacements of about 4–5 Å of the side chains with respect to the native antibody (not shown). Anyhow, the possible rearrangement did not affect the interaction with C3b. We then studied single antibody mutants (Ser-134, Thr-135, and Ser-136) to confirm the data obtained with the STS456 antibody. As can be observed in Fig. 5*A* none of the antibodies mutated in one single position showed any decrease in the intensity of band B. In the case of the Ser-136 mutant, the intensity of band B was even greater than that of chAb (control). Thus, these data confirm the results obtained with the STS456 mutant, indicating that none of these residues is responsible for the covalent anchorage of C3b.

When double mutants at positions 131–132 were studied, it was observed (Fig. 5*B*) that SS12 mutant antibody has an impaired ability to bind C3b, as shown by a significant decrease in the intensity of band B, suggesting the abolition of the C3b binding site in this mutant. This decrease is equivalent to that displayed by scAb in comparison with the chimeric antibody.

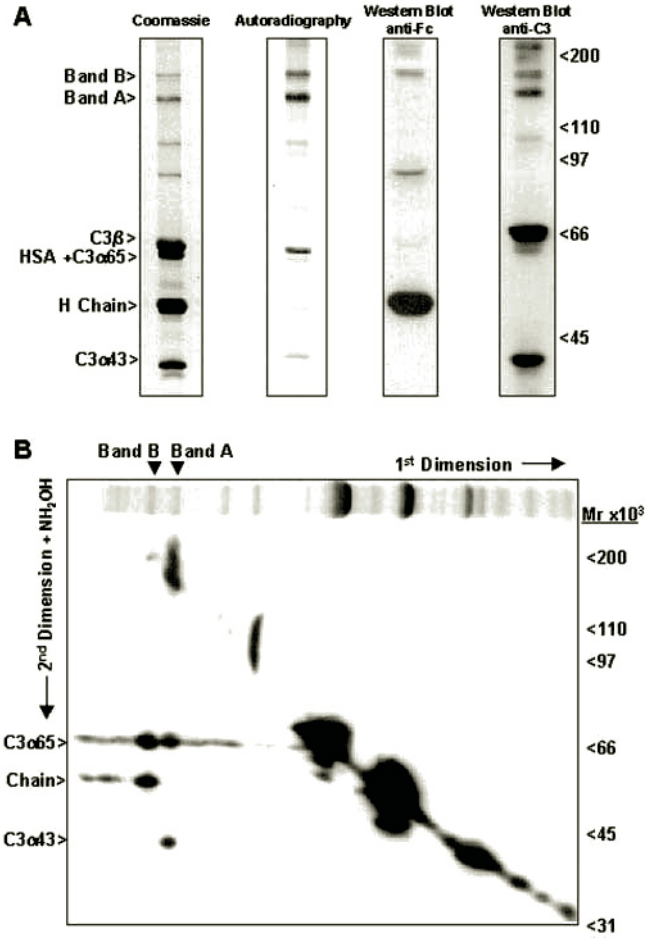

FIG. 4. *A*, identification of C3·chAb covalent adducts by SDS-PAGE under reducing conditions (*Coomassie lane*), autoradiography, and Western blot. *B*, two-dimensional SDS-PAGE analysis (stained gel) of C3b·chAb covalent complexes shown in *A*, after treatment with 1 M hydroxylamine. The molecular mass of markers is indicated on the right.

The analysis of C3b binding to single, individual mutants, at position 131 (Ser-131:Ser-131) and 132 (Ser-132:Ser-132) was performed to identify which residue was responsible for the diminished C3b binding in SS12 mutant. The decreased intensity of band B observed with the Ser-132 mutant is similar to that previously observed with the mutant antibody SS12 (Fig. 5*B*), whereas in the case of mutant Ser-131 the intensity of band B was not significantly altered. These data indicate that the mutation at position 132 is responsible for the lack of activity in the SS12 double mutant and strongly supports Ser-132 as the major C3b attachment point in this region of the CH1 domain.

We then examined two mutants at multiple positions, SS12STS456, and the latter also included position 139 (SS12STS456T9). Both showed a decreased band B similar to that of double (SS12) or single (Ser-132) mutated Abs (Fig. 5*C*), corroborating the importance of Ser-132 in the covalent attachment of C3b. When similar experiments were performed with antibodies mutated at position 139 (mutant T139A), no decrease was observed in band B, indicating that this residue was not involved in C3b binding (Fig. 5*C*).

C3b Covalent Binding to F(ab′)$_2$ Fragments of Mutant Antibodies—C3 covalent binding to F(ab′)$_2$·IC was studied in the same way as with complete Abs. A comparison of the formation of high molecular mass bands on SDS-PAGE of F(ab′)$_2$·IC with the chimeric IgG·IC is shown in Fig. 6*A*. Band A appears in the same position in both cases, whereas a new band, B′, was detected at about 100 kDa with the F(ab′)$_2$·IC. The two-dimensional gel (Fig. 6*B*) showed that band A dissociates into two spots of 65 and 43 kDa, and band B′ dissociates into two components of 65 and 23 kDa. Thus, band A is the same as that observed with IgG (C3α65·C3α43), and band B′ corresponds to the C3α65·Fd fragment complex. Figs. 6, *C* and *D*, shows the formation and quantitation, respectively, of band B′ using the F(ab′)$_2$ fragment from several mutants. It can be observed that band B′ almost totally disappears (>95%) from the F(ab′)$_2$ fragments mutated at position 132. In contrast, no variations are detected in the other F(ab′)$_2$ mutants. Thus, data from F(ab′)$_2$ fragments confirm those obtained with the complete mutant antibodies and indicate that Ser-132 is the major binding site of C3b on the Fab region of IgG. The putative binding of C3b to V domains has repeatedly been suggested (25, 26), although direct experimental evidence is still lacking.

DISCUSSION

In this work the covalent attachment point of C3b to the CH1 domain of human IgG1 has been determined by means of mutant antibodies. Using a chimeric antibody (V mouse, C human) as reference, we introduced mutations that result in the substitution of Ser and Thr residues by Ala. The absence of hydroxyl groups rules out any possibility of forming ester bonds with C3b. All the mutations were restricted to the first loop of the CH1 domain (residues 125–147), previously identified as a C3b binding region (10, 11). The analysis of binding assays with the mutant Abs showed an intense decrease of C3b binding to those Abs that lack the hydroxyl group at position 132 (Fig. 5). These data highlight the importance of this Fab region (125–147) in C3b binding and the key role of Ser-132. All the mutants containing this substitution (Ser-132 → Ala) showed a similar decrease in the intensity of band B. Mutation at Ser-132 produces an effect similar to the removal of the entire CH1 domain, because the amount of C3b bound by the mutant is equivalent to that of scAb. The binding ability of the remaining mutant Abs, which have Ser-132, is similar to that of chimeric Ab (Fig. 5).

Replacing Ser and Thr (polar residues) by Ala (apolar) produces only very small conformational alterations in the loop, since the gross conformation of the CH1 is dominated by the β-strands (27). Ser-134, Thr-135, and Ser-136 are situated in the center of the loop; therefore, their substitution by Ala would affect the conformation of the loop to a great extent than the residues situated around the edges (Ser-131, Ser-132, or Thr-139). A maximum displacement of 5 Å in the hydroxyl of Thr-135 was calculated by modeling these mutants. However, mutant Abs in the central positions (134–136) have not altered their ability to interact with C3b (Fig. 5, *A* and *B*), ruling out the participation of these residues in binding to C3b. In contrast, the conformation of residues close to the β-sheet (Ser-131, Ser-132, and Thr-139) are not predicted to be altered by the mutations. Consequently, the loss of ability to interact with C3b, observed in mutant at Ser-132, should be exclusively due to the lack of the hydroxyl group in this residue. Thus, the association between Ser-132 and the decreased intensity of band B identifies this Ser as the attachment point of C3b within the loop.

Sahu and Pangburn (12) synthesized a series of peptide analogs of the binding region (positions 125–147) to identify the residue favored for C3b attachment. The parent peptide included all six hydroxyl-containing amino acids present in the proposed binding site. Site-specific amino acid substitution of Thr and Ser residues in the peptide indicated little or no attachment occurred at Ser residues, and Thr-135 was proposed as the main residue involved in C3b covalent binding to IgG. However, this could be the consequence of the higher

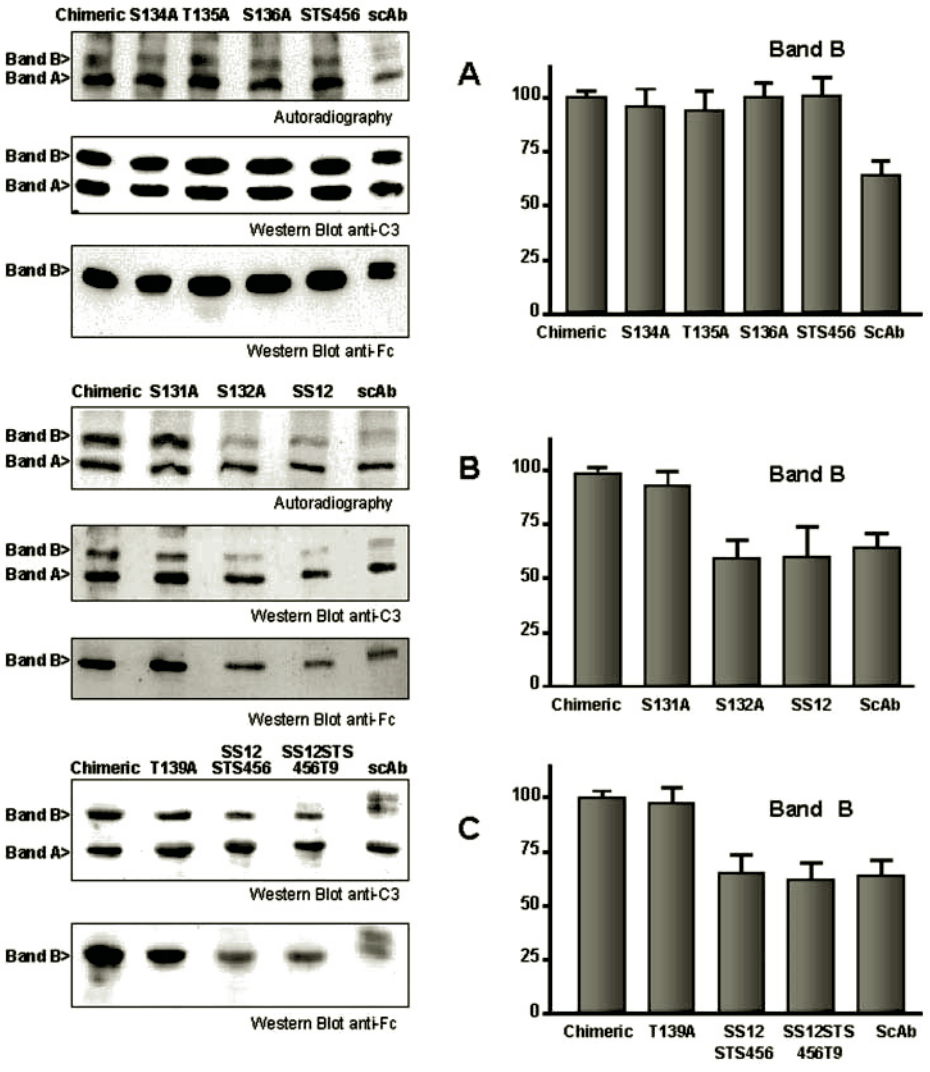

FIG. 5. **Analysis by autoradiography and Western blot of the C3b binding to mutant antibodies (*left panels*).** Bands were scanned and quantitated using the ImageQuant 3.1 software. Each *bar* (*right panels*) represents the average and the S.D. of a minimum of three independent assays.

reactivity of Thr residues in comparison with Ser (28, 29). All the peptides were small (4–11 amino acids), and only the largest spanned the complete region, with an affinity 20-fold less than for the IgG1 (12). In solution these peptides can adopt multiple unrestricted conformations that presumably do not mimic the very characteristic shape of the loop that contains the Ser-132 in the native conformation of IgG (Fig. 2).

The reactivity of the thioester group of C3b has been studied with numerous small molecules (30). This group is located in an environment of restricted access that conditions the specificity of C3b with its targets. C3b binding to the CH1 domain of the Ab takes place in a region with several Ser and Thr residues exposed to the solvent, which might facilitate the interaction of both proteins. However, only Ser-132 is the site of anchorage, even when there is another Ser in the adjacent position (Ser-131). Ser-132 is located at the end of the β-strand A (strand A-loop-strand B; Fig. 2), and its conformation is dependent on the structure of the β-sheet. The side chain of Ser-132 is totally exposed to solvent in a fixed position. This can favor its interaction with C3b in comparison with the other hydroxylated residues in the center of the loop, which have more freedom to move. In contrast, Ser-131 is only partially exposed (Fig. 7). The position of Ser-132 could explain the fact that C3b binds much more efficiently to IgG in the ICs than to free, monomeric IgG. In solution, Ab molecules have a marked segmental flexibility, which permits the Fab arms to move freely (to wave and rotate). However, once the interaction with the Ags has taken place, a three-dimensional lattice is formed which fixes the position of the Fabs. This generates a high concentration of C3b acceptor sites on the IC that can favor the interaction with C3b. The high local concentration of C3b binding sites on the ICs and the lack of mobility could be factors that favor the binding of C3b to ICs. C3b is able to bind covalently to free monomeric IgG but only when the Ab concentration is extremely high, as occurs in the case of infusion of intravenous immunoglobulin (31). Ser-132 is situated in the Fab, very near to the hinge region (32), in the groove generated by the interaction of the CH1-CL domains (Fig. 7). In IgG1, this position is adjacent to the Cys-220 (32) involved in the formation of the inter H-L disulfide bridge. In the other IgG subclasses (2, 3, and 4), Cys-131 (33) is the residue implicated in the disulfide bond with the L chain. This particular position of Ser-132 can explain previous data demonstrating the lack of ability to activate the alternative pathway of complement by IgG molecules with the inter H-L bond reduced (14, 34). Probably the cancellation of this disulfide bond disturbs this region, modifying the accessibility of Ser-132, impairing its interaction with the carbonyl group of the thioester of C3b. In addition, the interaction of C3b with Ser-132 could help explain the effects of C3b on the solubilization of ICs. This binding region (positions 125–147) is adjacent to residues 148–150, a component of a ball and socket point that exists between V and C domains. This structure has been hypothesized to modulate the V-C flexibility of the Fab (36) and has recently been shown to be an important functional

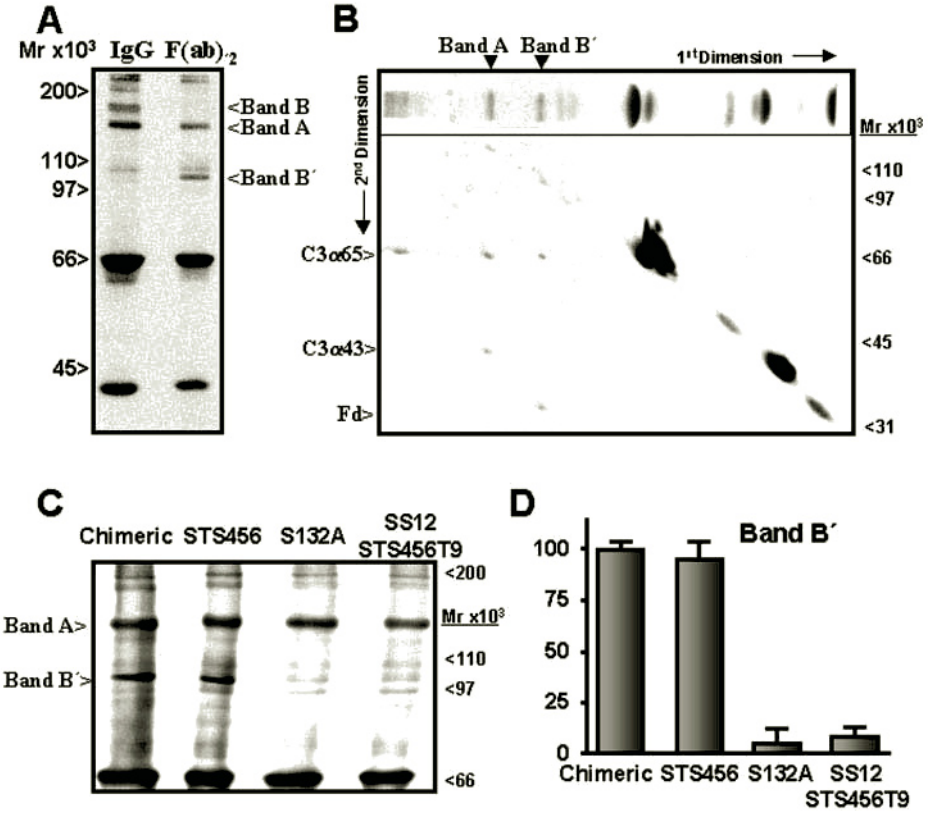

FIG. 6. *A*, SDS-PAGE analysis, under reducing conditions (stained gel), of C3·chAb and C3·F(ab′)$_2$ fragment covalent complexes. The position of bands A, B, and B′ are indicated. *B*, two-dimensional electrophoresis analysis of C3b-F(ab′)$_2$ covalent complexes shown in *A* after treatment with hydroxylamine. Band A dissociates into two spots of 65 and 43 kDa as in the case of the complete chAb. Band B′ dissociates into two spots of 65 and 23 kDa, which correspond to C3α65 and Fd fragment (N-terminal half of H chain: VH and CH1 domains). The assignment of the 23-kDa spot as Fd was carried out by blotting using an anti-CH1 domain monoclonal antibody (MCA1127G; Serotec). *C*, analysis of bands A and B′ of C3b·F(ab′)$_2$ fragment covalent complexes of chAb and different mutants. *D*, quantitation of band B′of the gel shown in *C*.

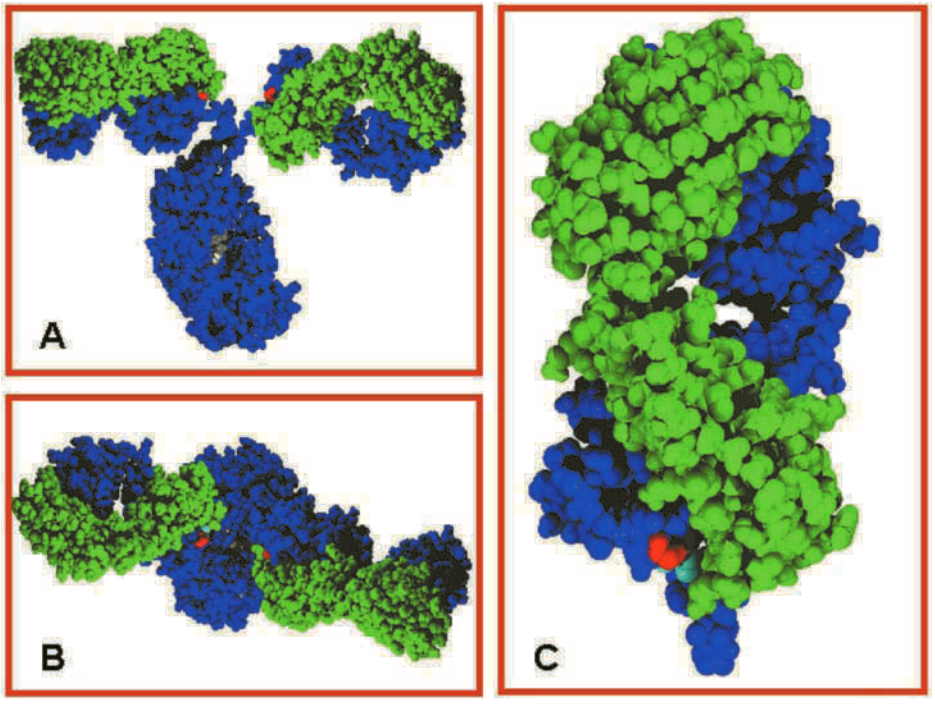

FIG. 7. *A* and *B*, two different views (front and zenital) of the structure of a complete rat IgG1 (PDB1IGY) molecule showing Ser-132 (in *red*); H and L chains are shown in *blue* and *green*, respectively. *C*, structure of a human IgG1κ Fab, showing the position of Ser-132 (in *red*). Ser-131 is partially exposed to solvent, and it is shown in *light blue*.

element of Ab structure (37). Covalent binding of C3b to this region can modify the V-C flexibility of Fabs in the ICs and facilitate their disruption. Furthermore, C3b is a large molecule (about 180 kDa) that, if bound to the CH1 domain, could interfere with Ag·Ab binding and with the Fc-Fc interactions, preventing the formation of the Ag·Ab lattice and leading to its disaggregation. The anchorage of C3b directly on the Fc regions (1, 8) would enhance the solubilizing effect.

Recent developments in genetic engineering now allow the production of designer antibodies of the desired specificity (38). However, for use in therapy, Ab effector functions need to be controlled according to the specific use of each Ab. The majority of these functions (complement activation, interaction with Fc receptors, catabolism, etc.) reside in the Fc portion of IgG and have been manipulated genetically to eliminate (39, 40) or enhance them (35). The present work adds a novel possibility for the design of Ab molecules, allowing the abolishment or attenuation of the main function known to reside in the CH1 domain. The replacement of Ser-132 by Ala abolishes the ability of the Fab region to bind C3b and to activate the alternative

pathway of complement. This kind of modification, by point mutation, is highly advantageous because it neither alters the CH1 domain nor the effector functions expressed by the Fc region. Work is in progress to identify the Fc residues involved in C3b covalent binding.

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