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The C4A and C4B Isotypic Forms of Human Complement Fragment C4b Have the Same Intrinsic Affinity for Complement Receptor 1 (CR1/CD35)

Liliana Clemenza*† and David E. Isenman2*†

Several previous reports concluded that the C4b fragment of human C4A (C4Ab) binds with higher affinity to CR1 than does C4Bb. Because the isotypic residues, 1101 PCPVLD and 1101 LSPVIH in C4A and C4B, respectively, are located within the C4d region, one may have expected a direct binding contribution of C4d to the interaction with CR1. However, using surface plasmon resonance as our analytical tool, with soluble rCR1 immobilized on the biosensor chip, we failed to detect significant binding of C4d of either isotype. By contrast, binding of C4c was readily detectable. C4A and C4B, purified from plasma lacking one of the isotypes, were CIs converted to C4Ab and C4Bb. Spontaneously formed disulfide-linked dimers were separated from monomers and higher oligomers by sequential chromatographic steps. The binding sensorgrams of C4Ab and C4Bb monomers as analytes reached steady state plateaus, and these equilibrium data yielded essentially superimposable saturation curves that were well fit by a one-site binding model. Although a two-site model was required to fit the equilibrium-binding data for the dimeric forms of C4b, once again there was little difference in the \( K_d \) values obtained for each isotype. Independent verification of our surface plasmon resonance studies came from ELISA-based inhibition experiments in which monomers of C4Ab and C4Bb were equivalent in inhibiting the binding of soluble CR1 to plate-bound C4b. Although divergent from previous reports, our results are consistent with recent C4Ad structural data that raised serious doubts about there being a conformational basis for the previously reported isotypic differences in the C4b-CR1 interaction. The Journal of Immunology, 2004, 172: 1670 –1680.

The immune adherence receptor mediates attachment of C3b- and C4b-opsonized particles to primate erythrocytes and phagocytic cells (1, 2). The protein responsible for this activity, subsequently named complement receptor 1 (CR1),3 consists in its most common variant form of 30 extracellular repeating modules, alternatively referred to as short consensus repeat (SCR) or complement control protein domains (3, 4). Based on internal sequence homology, the N-terminal-most 28 domains may be further subdivided into four long homologous repeats (LHRs), A, B, C, and D, each one consisting of seven SCR domains. Initial ligand-mapping studies indicated that C4b binding was localized to LHR-A, whereas LHR-B and LHR-C each had a binding site for C3b (5–7). It was further determined that most of the binding energy was contributed by the first three SCR domains of each LHR, although the first four domains were required to yield binding activity equivalent to the full-length LHR segment (7, 8). The C4b binding site in SCR domains 1–3 became known as functional site 1, and the C3b binding sites in the nearly identical three domains of SCR 8–10 and SCR 15–17 of LHR-B and LHR-C, respectively, each became known as functional site 2. It later became clear that site 2 was also capable of binding C4b (9, 10), although there is dispute about whether the site 2 interaction with C4b is weaker (9) or stronger (10) than that of C4b with site 1.

In humans, C4 is encoded by two distinct genes, C4A and C4B. Although the resulting mature heterotrimERIC proteins consisting of 93-kDa \( \alpha \)-, 75-kDa \( \beta \)-, and 33-kDa \( \gamma \)-chains are >99% identical with each other, the C4A and C4B isotypic variants are defined by the presence of one of two hexapeptide sequences, 1101 PCPVLD1106 and 1101 LSPVIH1106 for C4A and C4B, respectively (mature human C4 numbering used throughout). These residues are located in the central C4d fragment (~42 kDa) of the \( \alpha \)-chain (~100 aa C-terminal to the thioester-forming residues, the latter mediating covalent attachment of nascently activated C4b fragment to the C1-bearing target surface. Each of the isotypes displays allelic polymorphisms, with the C4d portion of \( \alpha \)-chain being the most polymorphic segment of the molecule (11). Included in these are residues at four positions (1054, 1157, 1188, and 1191), which together with those in the 1101–1106 isotypic sequences, give rise to the Rodgers (Rg) and Chido (Ch) blood group alloantigens. Generally, the Rg-determining polymorphic residues segregate with the C4A isotypic residues, while Ch-determining residues segregate with the C4B isotypic residues, but there are rare exceptions to this rule (12).

The most profound functional difference between the isotypes is in the nature of the covalent bond formed between the isotypic C4b fragments and the target surface, specifically an amide bond in the case of C4Ab and an ester bond in the case of C4Bb (13, 14). This is in turn controlled by the residue at position 1106, with histidine dictating ester bond formation, whereas virtually any other amino acid is capable of forming an amide bond. The principal difference between the isotypes is in the nature of the covalent bond formed between the isotypic C4b fragments and the target surface, specifically an amide bond in the case of C4Ab and an ester bond in the case of C4Bb (13, 14). This is in turn controlled by the residue at position 1106, with histidine dictating ester bond formation, whereas virtually any other amino acid is capable of forming an amide bond.
acid will result in amide bond formation (15–17; mechanism reviewed in Ref. 18). The nucleophilic preferences of the respective isotypic C4b fragments have been invoked to explain the greater transacylation efficiency of C4Ab to amino group-rich immune complexes vs the greater transacylation efficiency of C4Bb to carbohydrate-rich cellular target surfaces (14, 15, 19). The higher transacylation efficiency of C4Ab to immune complexes has in turn been used to explain the greater ability of native C4A, compared with C4B, to inhibit immune precipitate formation in serum or in serum-free mixtures containing Ag, IgG Ab, C1, and C4 (20, 21). Other functional properties, including cleavage rate by C1s, classical pathway C3 convertase subunit activity, regulation by complement factor I (fI) in the presence of C4b-binding protein (C4BP), and ability to act as a transacylation acceptor for C3b in the formation of the classical pathway C5 convertase, were found not to differ substantially between C4A and C4B (13, 14, 22). However, there have been three reports concluding that C4Ab (or, in one case, ammonia-treated C4A, a thioester-cleaved C4b-like species) displayed higher binding to CR1 than did C4Bb, or ammonia-treated C4B (23–25). The study by Reilly and Mold (25) used isotypic forms of covalent dimers of human C4b fragment, in which the cross-link was via the liberated thioester cysteine, to actually measure equilibrium-binding constants. They determined that dimers of C4Ab displayed a ∼4-fold higher affinity for CR1 than did dimers of C4Bb.

One reason for the interest in the functional properties of C4, and in particular of each isotype, is the apparent protective effect of C4 with respect to susceptibility to the autoimmune disease systemic lupus erythematosus (SLE; reviewed in Ref. 26). In humans, there is a 75% prevalence of SLE with complete C4 deficiency states of C4 are quite rare, in healthy white individuals, homozygous C4A and C4B deficiencies occur with an estimated frequency of 4 and 1%, respectively (26). Although not seen in every SLE patient cohort (e.g., Refs. 27 and 28), there have been multiple reports spanning two decades of an increased frequency of complete, or even partial, C4A, but not C4B, deficiency states in SLE patient cohorts as compared with healthy controls (29–37). The association of C4A null alleles with increased risk for SLE transcended ethnic boundaries, and thus was not readily attributable to the association of the C4A null allele to an extended MHC haplotype.

There are two nonmutually exclusive hypotheses for explaining the strong association of early classical pathway component deficiencies and SLE. First, C4 may have a role in the induction of self-tolerance against autoantigens (38, 39). Second, C4 has a role in the disposal of immune complexes and apoptotic bodies, the latter being loaded with precisely the autoantigens that one sees Abs against in SLE patients (26). The deposition of C4b fragment onto the target both provides the subunit for classical pathway C3 convertase formation and acts as a direct ligand for CR1, both of these events being important for the complement-facilitated disposal process. At present, nothing is known about the relative transacylation efficiency of C4Ab vs C4Bb to apoptotic bodies. However, both the greater ability of C4Ab relative to C4Bb to bind covalently to IgG, and thereby inhibit large and insoluble immune complexes from forming (20, 21), as well as the reported 3- to 4-fold higher affinity of C4Ab for CR1 (25) can be envisaged as preferentially facilitating the clearance of C4Ab-opsonized autoantigens. This, in turn, has been invoked as an explanation for the SLE association with C4A, but not C4B deficiency states.

Because the isotypic residues reside within the C4d region of the molecule, one might expect a contribution of this region to the binding interaction with CR1 in a way that reflects the isotype-defining sequence differences. Specifically, the isotypic residues may either be involved in direct contact with the receptor, or they may affect at a distance the conformation of a contact region in another part of the C4d molecule. With respect to the latter possibility, the presence of an additional proline (P1101) in the C4A isotypic sequence appeared to be a promising candidate to mediate such a conformational effect. However, the recently determined x-ray crystal structure of human C4Ad fragment did not support either one of these explanations (40). First, the loop containing the isotype-defining residues is in very close proximity to the site of covalent attachment, and as such, these residues are unlikely to be accessible for direct contact by CR1. Second, although there currently is no structure for C4Bd, based on the superimposability of the C4Ad structure on that of C3d, which is C4B-like in its isotypic segment and thus lacks a proline in the first position, we have previously argued that in all likelihood its backbone conformation will be indistinguishable from that of C4Ad (40). This would then render problematic the long-range conformational explanation for the isotype-dependent differences in the binding of C4b to CR1. There remained the possibility that the CR1-binding differences reflect contact differences with the major Ch/Rg epitopes defined by residues 1157, 1188, and 1191. As mentioned above, these normally segregate with the isotype-defining residues and, as visualized in the C4Ad structure, they should be highly accessible (40). In view of the various questions raised by the structure of C4Ad about the basis for reported CR1-binding differences of C4Ab and C4Bb, as well as the possible relevance of this differential affinity to our understanding of the etiology in humans of SLE, we decided to re-examine this issue. Specifically, we have used both surface plasmon resonance (SPR) and ELISA techniques to determine whether there is indeed an intrinsic affinity difference between C4Ab and C4Bb for a soluble recombinant form of CR1 (sCR1). Additionally, we have assessed the respective abilities of isolated C4c and isotypic variants of C4d fragments to bind to sCR1.

Materials and Methods

Purified proteins

Human rsCR1 was a gift from Avant Immunotherapeutics (Needham, MA), and human C1s was purchased from Advanced Research Technologies (San Diego, CA). C4A and C4B were isolated, as previously described (41), from plasma lacking one of the two isotypes. Because these proteins had been stored at −70°C for long periods of time, they contained a significant amount of thioester-hydrolyzed C4(H2O) material that would not be cleavable by C1s (42). Accordingly, these materials were repurified on a Mono-Q HR 10/10 FPLC column (Amersham-Pharaccia, Baie d’Urfé, Quebec, Canada), essentially as described by Hessing et al. (43), a procedure that is able to separate native C4 from C4(H2O). The purified proteins were converted to C4Ab and C4Bb, respectively, by overnight digestion at 37°C with 100 μl/well C1s. The digestion mixture was then chromatographed on a Mono-Q HR 10/10 FPLC column, as described previously, by a means of separating monomer C4b from spontaneously formed disulfide-linked dimer C4b (43). Briefly, using a flow rate of 1 ml/min and a starting buffer consisting of 50 mM NaCl, 20 mM Tris-HCl, and 2 mM EDTA, pH 7.4, the proteins were eluted from the Mono-Q column with a 50 ml linear gradient to a final concentration of 0.5 M NaCl. C4b dimers and monomers were collected as separate fractions and then dialyzed against PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.2). Following their respective concentration using Biomax Ultrafree-4 10-kDa cutoff spin concentrators (Millipore, Bedford, MA), each fraction was subjected to gel filtration on an FPLC Superose 6 HR 10/30 column (American-Pharaccia) equilibrated with PBS containing 0.02% NaN3, and running at a flow rate of 0.3 ml/min. Before use in BiAcore experiments, the proteins were dialyzed into 10 mM HEPES, 75 mM NaCl, and 3 mM...
EDTA, pH 7.4, and then surfactant P-20 (BIACore, Piscataway, NJ) was added to a concentration of 0.01%.

C4c was obtained from purified pooled C4 (i.e., containing a mixture of both C4 isotypes) that was digested first with C1s, as above, and subsequently with factor I (1:30 w/w) and C4BP (1:50 w/w) for 18 h at 37°C. Factor I (44) and C4BP (45) were purified from human plasma, as described previously. C4c was then purified by the same two-step chromatographic procedure described above for C4b and exchanged by dialysis into 10 mM HEPES, 75 mM NaCl, and 3 mM EDTA, pH 7.4, buffer, and for BIACore experiments, surfactant P-20 was added to 0.01%.

Recombinant C4Adg and C4Bdg fragments, differing only with respect to their 1101–1106 isotype-specific residues, were produced in Escherichia coli, as previously described (40). Cell lysates were loaded onto a 100-mL DEAE-Sephacel column (Amersham-Pharmacia) equilibrated with 10 mM Na phosphate, 25 mM NaCl, 2 mM EDTA, and 0.1 mM DTT. The purity of all proteins was assessed by SDS-PAGE. The extinction coefficients (ε280 nm) used were 8.2 for C4Adg/C4Bdg and C4c and 13.6 for C4Adg/C4Bdg.

**SPR measurements**

The respective interactions of C4Ab and C4Bb with sCR1 were analyzed by SPR technology on a BIACore X instrument (BIACore). The experiments were performed at room temperature in a half physiologic ionic strength buffer (10 mM HEPES, 75 mM NaCl, 3 mM EDTA, and 0.01% surfactant P-20, pH 7.4). sCR1 was diluted in 10 mM Na acetate, pH 5.5, at a concentration of 20 μg/ml and immobilized on flow cell 1 of a CM5 chip (BIACore) by using the amine-coupling kit from BIACore. Flow cell 2, to which mouse IgG (Sigma-Aldrich, St. Louis, MO) was immobilized using the same coupling conditions, was used as a control surface. A chip with 6800 resonance units (RU) on the sCR1 ligand surface and 6350 RU on the control surface was used to test the binding of C4Ab, C4Bb, and C4c fragments for most of the experiments shown in this study. A chip with 4900 RU on sCR1 ligand surface and 4400 RU on the control surface was used to test the binding of the isotypic C4dg fragments. The various C4 fragment protein analytes were used immediately after their last purification step. Binding was measured by injecting over the ligand-coupled chip several concentrations of analyte at a flow rate of 2 ml/min for 90–100 s. Following 180 s of buffer flow for dissociation, the sensor chip was regenerated with 1 M NaCl. The interaction of the C4dg fragments with sCR1 was also studied by reversing the immobilized ligand-analyte combination. Specifically, two different chips were generated by immobilizing C4Adg and C4Bdg to the respective sample flow cells (50 μg/ml in 10 mM Na acetate, pH 4.7, −1900 RU), and the control flow cells were in this case simply sham activated and then ethanolamine deactivated.

Equilibrium phase-binding data were fit to one- and two-site models of the Langmuir binding isotherm using the nonlinear regression program MacCurveFit 1.5 (K. Raner Software, Victoria, Australia). The equations describing these models were respectively:

\[
\Delta RU = \frac{\Delta RU_{max} \times [\text{analyte}]}{K_D + [\text{analyte}]} \quad (\text{Equation 1})
\]

\[
\Delta RU = \frac{\Delta RU_{max1} \times [\text{analyte}]}{K_{D1} + [\text{analyte}]} + \frac{\Delta RU_{max2} \times [\text{analyte}]}{K_{D2} + [\text{analyte}]} \quad (\text{Equation 2})
\]

where for Equation 2, the numerical subscripts refer to the equilibrium dissociation constant (K_D) and maximal resonance units signal change at saturation (ΔRU_{max}) associated with each class of binding site. The steady-state plateau-derived binding curves were also displayed as Scatchard transformations having the following forms for the one-site (Equation 3) and two-site (Equation 4) models, respectively (46):

\[
\frac{\Delta RU_{[\text{analyte}]} = K_D \times \Delta RU_{max} - K_D \times \Delta RU} \quad (\text{Equation 3})
\]

\[
\Delta RU_{[\text{analyte}]} = 0.5 \times (K_{D1} \times \Delta RU_{max1} - \Delta RU) + (K_{D2} \times \Delta RU_{max2} - \Delta RU) + \sqrt{(K_{D1} \times \Delta RU_{max1} - \Delta RU)^2 - 4 \times K_{D1} \times \Delta RU_{max1} \times \Delta RU} \quad (\text{Equation 4})
\]

where for Equation 4, the numerical subscripts refer to the equilibrium association constant (K_A) and maximal response units signal change at saturation (ΔRU_{max}) associated with each class of binding site. Kinetic data were analyzed globally using the BIAevaluation 3.0 software package. The binding model used for the C4b and C4c monomer sets of sensorgrams was the conformational change model: A + B ↔ AB ↔ AB* (analyte (A) binds to ligand (B); complex AB changes to AB*, which cannot dissociate directly to A + B). The binding model used for the C4b dimer curves was the parallel binding model for a two-site heterogeneous binding model: A + B ↔ AB1; A + B2 ↔ AB2 (the binding curve obtained is the sum of the two independent reactions).

**ELISA-based inhibition-binding assay**

ELISA plates were coated with 100 μl of 10 μg/ml mixed-isotype C4b fragment. In a preliminary experiment in which variable concentrations of sCR1 were added to the C4b-coated wells, we established that ~50% of maximal binding occurred at a concentration of 1 μg/ml sCR1. For the inhibition experiments, this concentration of sCR1 was preincubated for 1 h at room temperature with varying concentrations of C4Ab/C4Bb monomer (from 200 to 3.125 μg/ml) in a total volume of 100 μl. Uncomplexed sCR1 in each preincubation mix was then allowed to bind to plate-bound C4b (1 h, room temperature), and its binding was detected by using the anti-C4 Ab E11 (BD Pharmingen, Mississauga, Ontario, Canada) as the primary Ab and an anti-mouse IgG conjugated to alkaline phosphatase as the secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). The assay was performed in 1/3 ionic strength PBS (10 mM Na phosphate, 50 mM NaCl, and 0.02% NaN3, pH 7.2).

**Results**

SPR assessment of the interaction of the C4d and C4c fragments with CRI

Given that the C4d fragment forms an independently folded domain entity (40) and the indications from the literature (23–25) that the C4d-localized isotypic segment in some way affects the interaction of the parent C4b molecule with CR1, we first wished to determine whether C4d on its own displayed any binding activity for CR1, and if so, whether there were isotype-dependent differences in binding activity. For these experiments, we have used rC4d fragments corresponding in length to the physiologic fI cleavage-derived product (residues 938-1317), but, by analogy with the C3 fragment nomenclature, we have named them C4Adg and C4Bdg. This is to distinguish them from the N-terminally truncated C4d portion of the molecule that is visible in the x-ray structure and that corresponds to length in the C3d proteolytic limit fragment of fI-derived C3dg. We tested several concentrations of C4Adg or C4Bdg analyte (1–12 μM) for binding to biosensor-coupled sCR1, but found no binding signal above that seen on the control IgG-coupled flow cell. Shown in Fig. 1 are representative experiments performed at 9.5 μM for each of C4Adg and C4Bdg. Because within each CR1 molecule there are potentially three binding sites for C4b, and because the interaction of CR1 with an array of C4b molecules on a target is an avidity situation, we wished to determine whether binding of sCR1 would be observed to an array of C4dg molecules, as this better mimics the physiologic avidity situation. Accordingly, we reversed the analyte-ligand combination from the first experiment and tested the binding of several concentrations of sCR1 to biosensor-coupled C4Adg or C4Bdg. Once again, however, no specific binding was observed in either case (results not shown).
In view of the failure of isolated C4dg to bind to CR1, we next asked whether the complementary fl-generated degradation fragment of C4b, namely C4c, could on its own bind to CR1. As can be seen in Fig. 2, injection of variable concentrations of C4c analyte (see Fig. 3 for SDS-PAGE analysis of the C4c material) over an sCR1-coupled flow cell resulted in a dose-dependent and specific binding response. Because a steady state plateau was not reached for any of the concentrations of analyte tested, we were unable to perform a direct analysis of the equilibrium-binding data. Global kinetic analysis of the binding of C4c to sensor-bound sCR1 using the BIAevaluation 3 software package did not yield a satisfactory fit to a simple 1:1 binding model. However, an acceptable fit ($\chi^2 < 2$) was obtained using a model in which a conformational change followed an initial binding event, and from these kinetic parameters one could extract a value of 1.6 M for an estimated $K_D$ for the overall reaction (see Fig. 2 for further details).

**SPR assessment of the interaction of isotypic variants of C4b monomers and dimers with CR1**

Although the isolated C4dg fragments do not have detectable binding affinity for CR1 on their own, this region may still contribute to the binding of the parent C4b fragment, potentially in an isotype-dependent manner. We therefore next analyzed the binding of monomeric and dimeric forms of C4Ab and C4Bb to biosensor chip-coupled sCR1. As reported previously by Hessing et al. (43) for mixed isotype human C4, we observed that activation of either C4A or C4B by C1 resulted in 10–20% of the C4b material undergoing spontaneous disulfide-mediatedimerization. This most likely occurs via the cysteine residue liberated upon the thioester hydrolysis that accompanies the proteolytic cleavage event. In accordance with the published procedure, the monomer and disulfide-linked dimer populations were separated by ion exchange on an FPLC Mono-Q column, and Fig. 4 shows the elution profile for C1s-treated C4A. Also shown in this figure are the FPLC gel filtration profiles for each of the monomer and dimer peaks from the Mono-Q column. The elution profiles for the C4B-derived material were very similar (data not shown). The SDS-PAGE analysis for the purified monomer and dimer forms of C4Ab and C4Bb is shown in Fig. 3. It can be seen on the nonreduced gel that the dimer fractions contain relatively small amounts of contaminating monomer, and the level of contaminant is the same for both isotypes. The C4Bb monomer fraction appears to be totally devoid of dimer and C4Ab contains only a trace amount (as well as a trace amount of C4c). The relative paucity of α-dimer band(s) upon reduction indicates that the vast majority of the dimers are disulfide-linked, as opposed to ester or amide linked, which would

**FIGURE 1.** Lack of interaction of recombinant C4Adg and C4Bdg with sensor-bound sCR1. A concentration of 9.5 $\mu$M of analyte was injected for 90 s over a sensor chip. sCR1 was coupled to the experimental flow cell (4900 RU), and human IgG was coupled to the control flow cell (4400 RU). The C4dg curves are shown as dashed lines; the control curves as dotted lines; and the net curves, obtained by subtracting the respective control RU signals from the experimental RU curves, are shown in solid black. The arrows indicate the start and end points of the analyte injection.

**FIGURE 2.** Analysis of the interaction of C4c with sensor-bound sCR1. Sensogram overlays generated by injecting several concentrations of C4c (indicated at the right of the curves in decreasing order from the conditions of the uppermost curve) over a chip coupled with 6800 RU of sCR1. Here, and in subsequent BIAcore data panels, the curves shown represent the net binding after the subtraction of the combined nonspecific binding and bulk refractive index change on the control channel that had been coupled with 6300 RU of human IgG. The interaction was analyzed by global kinetic analysis using the conformational change model in the BIAevaluation 3.0 software package. The fitted curves are indicated with solid lines through the experimental time points. The conformational model is of the form:

$$A + B \rightleftharpoons AB \rightleftharpoons AB^*$$

(Equation 5)

where $k_{a1}$ and $k_{d1}$ are the forward and reverse rate constants for the encounter complex and $k_{a2}$ and $k_{d2}$ are the forward and reverse rate constants for the conformational change. The overall equilibrium $K_{eq}$ is given by the formula $(k_{d2}/k_{a2}) \times (k_{d1}/k_{a1})$ and was 1.6 M in this case. The individual rate constant values are as follows: $k_{a1} = 9.2 \times 10^7$ M$^{-1}$ s$^{-1}$, $k_{d1} = 0.24s^{-1}$, $k_{a2} = 6.8 \times 10^{-3}$ s$^{-1}$, $k_{d2} = 4.3 \times 10^{-3}$ s$^{-1}$.
represent thioester-mediated transacylation products. The material depicted on these gels is representative of the samples used in our SPR measurements, these measurements being performed as soon as possible after the Superose 6 chromatography. We did, however, note that upon storage at 4°C, dimer formation occurred in the purified monomer fraction and that this appeared to be more prevalent for the purified C4Ab monomers.

Depicted in Fig. 5A are the sensorgram overlay curves obtained for the interaction of the C4Ab and C4Bb monomer analytes injected over sensor-coupled sCR1 using an analyte concentration range of 0.06–1 μM. As can be seen readily for the first five incremental concentrations, in which the analyte concentrations were perfectly matched, the two isotypes of C4b monomer behaved in a very similar manner. Both displayed fast association and fast dissociation kinetics and, as for C4c, the satisfactory global fitting of the kinetic data required the use of a conformational model. As the sensorgrams reached a steady state plateau in all cases, we were able to directly analyze the equilibrium data by both nonlinear regression analysis of the saturation curves (Fig. 5B) and Scatchard transformation of the data (Fig. 5C). These analyses showed that the data for both C4Ab and C4Bb monomers were well fit by a simple 1:1 Langmuir binding model and that the two saturation curves were essentially superimposable. The Scatchard transformations similarly yielded nearly identical linear plots that were indicative of simple homogeneous binding. Although for both C4Ab and C4Bb the lowest concentration point deviated somewhat from linearity and may reflect a degree of biochemical heterogeneity in the binding reaction (see Discussion), the deviation could also be artifactual, as this part of a Scatchard plot is the most sensitive to deviations from ideal behavior as a

**FIGURE 3.** SDS-PAGE of the purified C4b and C4c fragments used for BIAcore analysis. The proteins were analyzed on an 8% gel under nonreducing conditions (top) and a 10% gel under reducing conditions (bottom). Molecular mass markers are indicated on the left.

**FIGURE 4.** Purification of monomers and dimers of C4b. C1s-digested C4A or C4B was first chromatographed on a Mono-Q column to separate monomers from spontaneously formed dimers (top panel). The proteins were eluted with a 50 ml NaCl gradient, as indicated. Monomer (middle panel) and dimer (bottom panel) fractions were further purified on a Superose 6 gel filtration column. The boundaries of the collected material for each peak are indicated. The data shown are for C4A, but were similar for C4B.
result of small errors in the measurements. As can be seen in Table I, both isotypes of monomeric C4b displayed virtually identical $K_D$ values of ~0.9 μM, and the total binding capacity, as defined by the respective $\Delta \text{RU}_\text{max}$ values, was similarly close.

A previous report that compared the binding of the isotypic forms of human C4b with red cell-associated CR1 used chemically cross-linked dimers of C4b (bismaleimidohexane cross-linking via the thioester cysteine) to take advantage of the avidity effect, and therefore enhanced readout signal, that dimerization provides (25). Accordingly, we also measured the binding of our spontaneously formed disulfide-linked dimers of C4Ab and C4Bb to biosensor-bound sCR1. As can be seen from the overlay plots shown in Fig. 6A, the C4Ab and C4Bb dimers once again showed very similar concentration-dependent binding behavior. This time, the best kinetic fit was obtained with a parallel binding model to two independent classes of binding site. Although we realize it to be somewhat of an approximation, we have taken the $\Delta \text{RU}$ values at the end of the injection phase to represent pseudo-steady state plateau values, as this allowed a comparison of equilibrium phase functional affinity constants for the dimer forms of C4Ab and C4Bb for CR1. Consistent with the kinetic analysis, the minimal model that could fit the pseudo-steady state saturation data was that having two classes of binding sites (Fig. 6B). This is most clearly indicated by the inverted hyperbola shape of the Scatchard-transformed and fitted data (Fig. 6C). Nevertheless, the values of the equilibrium dissociation constants extracted for each class of site are very similar for the two C4 isotypes, as is their fractional representation of $\Delta \text{RU}_\text{max}$ (see Table I for a summary of all the equilibrium-binding data). Specifically, ~13% of the binding sites are of high affinity and have a $K_D$ of ~10 nM, but the bulk of the

### Table I. BIAcore equilibrium state analysis of the interaction of sCR1 with C4Ab and C4Bb

<table>
<thead>
<tr>
<th>Monomers</th>
<th>1-site model$^a$</th>
<th>Dimers</th>
<th>2-site model$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_D$, μM</td>
<td>$\Delta \text{RU}_\text{max}$</td>
<td>$R^2$</td>
</tr>
<tr>
<td>C4Ab</td>
<td>0.93 ± 0.15</td>
<td>261 ± 25</td>
<td>0.993</td>
</tr>
<tr>
<td>C4Bb</td>
<td>0.94 ± 0.14</td>
<td>281 ± 23</td>
<td>0.990</td>
</tr>
</tbody>
</table>

$^a$Parameters fit according to Equation 1 of Materials and Methods; error estimates of the fit are indicated.

$^b$Parameters fit according to Equation 2 of Materials and Methods; error estimates of the fit are indicated.

$^c$For the 2-site binding model, reported $K_D$ values are based on concentrations of the C4b covalent dimer species and would be 2-fold higher if calculated based on the concentration of monomeric subunit.

$^d$Projected $\Delta \text{RU}$ change at saturation for each component of the binding, with the fractional numbers in parentheses being the individual component $\Delta \text{RU}_\text{max}$ divided by the sum of these for the two components.
binding is of lower affinity, having a $K_D$ of $0.4 \mu M$. Because the analyte concentrations were calculated on the basis of a dimer molecular mass of 380 kDa, if one calculates it on the basis of C4b subunits present, this latter $K_D$ value becomes very similar in magnitude to what we had observed for monomeric C4b binding to biosensor-coupled sCR1.

As a means of verifying our SPR results via an independent approach, we have used an ELISA-based inhibition assay in which the binding of sCR1 to plate-bound mixed-isotype C4b was evaluated after preincubation with variable concentrations of monomeric C4Ab or C4Bb as fluid-phase competitors. This assay format was chosen because any masking effect on C4b isotypic ligand preference brought about by the covalent coupling of sCR1 to the biosensor chip would not be a factor in this fluid-phase competition assay. As shown in Fig. 7, and in accord with the SPR data, monomers of C4Ab and C4Bb were equipotent in their inhibitory activity.

Discussion
In contrast to several previous reports in the literature (23–25), we find that there is no difference in the intrinsic binding ability of the isotypic forms of human C4b fragment to human CR1. As will be elaborated upon below, the basic conflict between our results and the earlier ones is not about the correctness of the earlier data, but rather that the higher binding to CR1 observed for C4Ab was being ascribed to an inherent property of its contact site for CR1. Instead we will argue that the higher binding of C4Ab observed was most likely due to effects that conferred on it a functional affinity/avidity advantage in a particular experimental system.
Intrinsic affinity generally refers to the equilibrium-binding constant between a monovalent ligand and an acceptor molecule having a single class of binding site for that ligand. So long as the acceptor sites are sufficiently separate so that there is no steric interference between them when ligand is bound, multivalency of the acceptor molecule will in principle not affect the observed affinity of the monovalent ligand. Although to the best of our knowledge there is no definitive biophysical proof on this point, it has been generally assumed that a single C4b molecule acts as a monovalent ligand for CR1. CR1, however, has three potential binding sites for C4b (9, 10), one in LHR-A (site 1) and one in each of LHR-B and LHR-C (site 2). There is evidence in the literature that a dimeric form of C4(CH$_3$NH$_2$)$_2$, a thioester-cleaved C4b-like molecule, can bind to sCR1 in solution in a monogamously bivalent manner, that is to two of the three potential C4b binding sites on the same CR1 molecule (47). There is, however, dispute about whether binding of C4b to site 1 has a relative affinity ~3-fold higher than that observed for site 2 (9) or whether the binding of C4b to site 2 is, if anything, ~2-fold stronger than that to site 1 (10). In our experiments in which monomeric C4Ab and C4Bb were injected over an sCR1-coupled biosensor chip, the data were well fit to a model having a single class of binding site. If there were significant contribution of a second class of binding site whose affinity differed by 3-fold or more from that of the first class, there should be more curvature in the Scatchard transformation of the data (Fig. 5C) than what was observed. Given that the reported differences in binding strength in either direction of the two sites are relatively small (9, 10), and also that for monovalent ligand there is a 2:1 ratio of site 2 to site 1 present in full-length sCR1, we believe that the most likely interpretation of the essentially homogeneous nature of the binding data observed is that there are no major differences in affinity for C4b between sites 1 and 2. Thus, the observed $K_{d}$ of ~0.9 μM for both C4Ab and C4Bb binding to intact sCR1 most likely reflects a weighted average affinity of collective binding to sites 1 and 2. However, because Reilly et al. (9) found that dimers of C4b bound to cells transfected with cDNA encoding full-length CR1 with the same affinity as to cells transfected with a cDNA encoding only site 1, and that in their hands this affinity was 3-fold higher than to cells bearing only site 2, we cannot totally rule out the possibility that the homogeneous binding that we observe is dominated by binding to site 1 only.

Because we were concerned that the surface effects inherent in the SPR technology might be obscuring subtle differences in intrinsic affinity of the isotopic forms of C4b for CR1, we also conducted an ELISA-based competition experiment that allowed one to monitor the binding of monomeric C4Ab and C4Bb to sCR1 in solution. In this instance, too, however, the respective binding behaviors of C4Ab and C4Bb to sCR1 were indistinguishable, thereby confirming the results obtained using the BIAcore instrumentation.

It is worth mentioning that in choosing to work with monomeric C4Ab and C4Bb so that we monitored intrinsic binding ability in both the BIACore and competition ELISA, the technical compromise that we had to make was to work at subphysiologic ionic strength. In this way, it was possible to achieve a meaningful degree of binding site saturation for what is well established to be a highly salt-sensitive interaction (10, 24). Although it is reasonable to have some concern about extrapolating our results to physiologic ionic strength, we note that in one of the earlier studies that reported higher sCR1 binding of a thioester-cleaved form of C4A relative to C4B, the authors stated that the binding difference was actually accentuated at low ionic strength (24).

Spontaneously formed disulfide-linked dimers of C4b, or even C4(CH$_3$NH$_2$)$_2$ (43, 47), have been reported previously, and presumably these form via the thioester cysteine. As expected, dimers of both C4Ab and C4Bb did show enhanced binding to biosensor-coupled sCR1 due to avidity effects, but in this instance too the magnitude of the effect was essentially the same for each C4 isotype. The high affinity component was ~100-fold greater than the affinity observed with C4b monomers (Table I). This is commensurate with the approximations made by others when they compared the relative ability of unlabeled monomers and dimers of C4b, or C4(CH$_3$NH$_2$)$_2$, to inhibit the binding of their labeled dimeric counterparts to the endogenous CR1 on human red cells (9, 47). What was somewhat unexpected was that higher avidity component accounted for a relatively small fraction (~13%) of the total binding, whereas the majority of the binding had a $K_{d}$ commensurate with binding of C4b monomers (Table I). Either the procedure used to couple sCR1 to the biosensor chip had made bivalent attachment a sterically unfavorable event, or the time course of the BIAcore experiment (100 s) is too short for a more substantial amount of bivalent binding to occur. With respect to the latter point, although it was done at a lower temperature than our experiment (0°C vs 22°C), it has previously been reported that binding of C4b dimers to red cell CR1 displayed very slow kinetics, taking 90 min to reach equilibrium (9).

Inferences drawn from the structure of C4Ad, and its comparison with the structure of C3d (40), had essentially predicted what we have observed experimentally, namely that the isotopic sequence differences should not influence the respective intrinsic CR1-binding affinities of C4Ab and C4Bb. The crux of the argument was that the backbone segments in and around the thioester, as well as the three-dimensionally proximal isotope-defining sequences, were completely superimposable. Yet, the C4A-specific sequence PCPVLVD was at least as dissimilar from the corresponding C3d segment DAPVVIH as it was from the C4B-specific isotopic sequence LSPVVIH. In particular, the extra proline of the C4A-specific sequence was without conformational consequence. Although there is no structure available for C4Bd, it is highly unlikely to be different at the level of backbone structure from that of C4Ad. Consequently, there is no conformational basis for the isotope-defining segment influencing a remote CR1 contact site in C4d. Furthermore, direct contact of the isotopic residues with CR1 is probably precluded by their proximity to the covalent attachment site. We know from previous serumotyping (48) that the C4A and C4B proteins that we used were of the common A3 and B1 allotypes, and that they had also been typed as being Rg"Ch" and Rg"Ch", respectively. Although the Ch/Rg epitope-determining residues at 1157, 1188, and 1191 are surface exposed in the C4d structure, and are well away from the site of covalent attachment, the equivalence of the intrinsic affinities of C4Ab and C4Bb for CR1 that we find shows that these residues also do not directly affect this interaction.

We believe that the difference between our results and the three previous reports claiming that C4Ab displayed a higher affinity for CR1 than did C4Bb can be attributed to issues related to the characterization of the ligands. In the study by Gatenby et al. (23), radioiodinated IgG-containing Ag-Ab complexes were opsonized in the presence of purified C1 with either C4A or C4B before their binding to CR1-bearing human red cells was determined. Although substantially higher binding of the radioiodinated immune complexes to CR1 was observed when C4A was the opsonin, the study made no correction for what was certain to be a higher deposition of C4Ab, than C4Bb, to the same amount of immune complex. Based on the experiments of Kishore et al. (19), for the same amount of IgG transacylation target and constant C1, C4A transacylates onto IgG H chain at a 3- to 4-fold higher efficiency than does C4B. Additionally, because the transacylation target nucleophiles within the immune complex are going to be different for
nascent C4Ab and C4Bb, the nature of the ligand clusters formed could affect the binding to CR1, which is itself clustered on red cells (49). Thus, the higher binding of C4Ab-opsonized complexes that was observed by Gatenby et al. (23) is most likely accounted for by the well-established covalent binding differences of the two human C4 isotypes.

The study by Reilly and Mold (25) compared the functional affinity of radiolabeled dimers of C4Ab and C4Bb to CR1 on human red cells and reported a ~4-fold higher affinity for dimers of the C4A isotype. These workers presumably chose to take advantage of the avidity properties of dimers because their binding assay, which included three wash steps, would not have yielded a sufficient binding signal with monomeric ligand. Reilly and Mold were well aware that the presence of higher molecular mass oligomers would bias their results due to an enhancement of the avidity effect. Consequently, they put their bismaleimidohexane-cross-linked proteins through a size exclusion chromatography step, which by their estimation reduced the proportion of higher molecular mass aggregates to less than 3%. The gel that they show of their covalent C4Ab and C4Bb dimers was run under reducing conditions, which would not break the bismaleimidohexane cross-links between α'-chains, but would reduce disulfide-linked oligomers. Based on our observations on the unique properties of C4Ab in forming disulfide-linked oligomers beyond the dimer stage, we strongly suspect the presence of such species uniquely within the aggregate portion of their C4Ab dimer preparations, but stage, we strongly suspect the presence of such species uniquely within the aggregate portion of their C4Ab dimer preparations, but these would only have been detectable on a nonreducing gel. Specifically, it has been our experience that C4Ab has a higher propensity to form spontaneous disulfide-linked dimers than does C4Bb. Additionally, on nonreduced, but not on reduced SDS-PAGE, the C4Ab dimer fraction from the Mono-Q column contains an additional higher molecular mass species that is not seen in the C4Bb dimer fraction (data not shown).

The study of Gibb et al. (24) used ammonia treatment of C4A and C4B to cleave the thioester bond and induce a C4b-like conformation. When equal amounts of ammonia-treated C4A and C4B were coated onto ELISA wells, it was found that the binding of 125I-labeled sCR1 was ~2-fold greater to the C4A material than to C4B. Because the ammonia treatment would liberate the thioester cysteine, as well as the isotypic cysteine of C4A, not only would there be spontaneous disulfide-linked dimers formed somewhat more preferentially for C4A, but as discussed above, higher oligomers would only be possible in the case of C4A. Such a cluster of C4Ab-like molecules may be better suited for capturing the sCR1 by binding simultaneously up to three sites on the same CR1 molecule, thus conferring an advantage relative to the wells coated with ammonia-treated C4B.

Although neither C4Adg nor C4Bdg on its own was able to bind to CR1, substantial binding was observed for C4c. This parallels the behavior of C3 degradation fragment binding to CR1, namely that C3c on its own binds to CR1, albeit with weaker affinity than C3b, whereas C3d shows no binding whatsoever to CR1 (50, 51). Because the sensorgrams of C4c binding to an sCR1-coupled chip did not reach a steady state plateau, it was only possible to extract binding affinity data though analysis of the kinetics. In our general experimental design, we were most interested in using the equilibrium plateau regions of the sensogram as a readout of the binding C4Ab and C4Bb monomers, as this method of analysis is not influenced by known kinetic artifacts of the BIAcore instrumentation. These involve limitations on mass transport to analyte binding sites within the carboxylated dextran matrix, which leads to an underestimation of $k_{on}$ values, and re-binding of ligand during the dissociation phase of the experiment, which leads to an underestimation of $k_{off}$ values. Because for the equilibrium-phase analysis one wishes to maximize signal change, the experiments were conducted at relatively high RU values (6800) of sensor chip-coupled sCR1 and also using relatively high analyte concentrations so that binding data extending through at least 60% of the saturation curve could be achieved. Unfortunately, these are precisely the conditions that exacerbate the problems in kinetic measurements. Accordingly, we put little credence into the absolute value of the kinetic constants, or the equilibrium constants derived from them. Nevertheless, because C4c and the monomers of C4Ab and C4Bb were analyzed on the same chip, and required the same conformational mechanism to kinetically fit the data, it may be valid to compare in relative terms the respective kinetically derived equilibrium-binding constants. Whereas for C4Ab and C4Bb, the kinetically derived $K_D$ values were respectively 0.26 and 0.31 μM (Fig. 5), that for C4c was 1.6 μM (Fig. 2), suggesting ~5-fold weaker binding relative to the parent C4b ligand. Given the complete absence of CR1-binding activity of C4dg on its own, we infer that the higher affinity of C4b for CR1 represents an indirect scaffolding effect whereby one or more contacts between the C4d domain and the larger C4c fragment may stabilize the conformation of CR1-contacting segments located within the C4c part of the C4b molecule.

The fact that the kinetic data obtained with monomers of C4c, C4Ab, or C4Bb could not be fit by a simple 1:1 encounter model, but rather required invoking a kinetically coupled unimolecular conformational change subsequent to the initial binding event, may also be meaningful in terms of the nuclear magnetic resonance structure of CR1 site 2 (SCR domains 15–17) and the mapping onto this structure the location of mutants that are deleterious to C3b or C4b binding (52). Whereas such mutations in SCRs 15 and 16 are localized on one face of the molecule, those in SCR 17 are located on the opposite face of the molecule and require a swivel about the SCR 16–17 linker segment to align with what is the presumed ligand contact face contributed by SCRs 15 and 16. If the initial contact of C4b is with the first two domains of either site 1 or 2, then the conformational component to the kinetic analysis may reflect the swivel required to enable the contact with residues in the third SCR domain contributing to the binding site.

The debate about the possible association of SLE with complete, or even partial, C4A deficiency states is at present not settled. It has been noted (11) that most of the early C4 population studies relied on C4 protein allotyping in the context of a two-locus per allelic chromosome genetic model. In these studies, the presence of a null allele was derived from the comparison of the relative protein levels of the two isotypes. Recent genetic studies have revealed a far more complex picture for the organization of the C4 gene. In the current model, the C4 gene is part of a module containing the RP-C4-CYP21-TNX genes (RCCX module) that can be present in single, double, triple, or even quadruple copies on each allelic chromosome. In turn, each C4 gene locus may encode either C4A or C4B protein (11). Therefore, precise C4 genotyping, including module-specific RFLP and PCR analysis to determine the...
exact number of C4 genes (53), is necessary in order not to mis-
interpret the overdosage of one isotype as a partial deficiency of
the other isotype. Nevertheless, even recent studies that have in-
cluded the appropriate genotyping at the DNA level continue to
show strikingly conflicting results on the association of C4A de-
iciency states and SLE (28, 37). Although we cannot resolve this
corroversy, if the selective lack of C4A does indeed contribute to
the development of SLE in at least some ethnic populations, our
results suggest that it is not due to differences in the intrinsic bind-
ing affinity between C4Ab and C4Bb for CR1. This being said, if
our in vitro observations on the greater propensity of C4Ab to form
disulfide-linked dimers, and its unique ability to form higher mo-
lecular mass oligomers via its isotypic residue C1102, were to also
occur in vivo, the clustering effect of the ligand may confer a
higher avidity interaction with CR1 for C4Ab-opsonized targets.
In keeping with an earlier suggestion (25), such a higher functional
affinity for CR1 of C4Ab-opsonized targets could enhance the ef-
fect due to the inherently higher covalent binding efficiency to
amo group-rich targets, such as immune complexes, of C4Ab
relative to C4Bb. These combined effects may therefore confer an
advantage to having high levels of C4A for the disposal of immune
complexes, and perhaps apoptotic bodies, a disposal process that is
thought to be instrumental in the etiology of SLE (26).

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