Characterization of the interactions of complement receptor 2 with its ligands iC3b, C3d and EBV glycoprotein gp350/220

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The complement system, a key component of innate immunity, is a group of serum and membrane-bound proteins and glycoproteins that participate in various aspects of the immune defense of the host. In this system, the presence of foreign molecules or immune complexes engages complex enzymatic cascades in which one complement component sequentially binds and activates another. Activation occurs through three distinct pathways; the alternative, classical and lectin pathways, depending on the nature of the activating surface and the recognition molecule involved. Assembly of the terminal components of these pathways on the target cells and microorganisms leads to membrane leakage and direct lysis. Moreover, during the course of activation of these cascades a number of biological processes are initiated, such as inflammation, phagocytosis, immune clearance, selection and enhancement of the organism's antibody repertoire and tolerance by the various complement components (Volanakis and Frank, 1998).

The third component of complement, C3, is the central component of this system. It is a soluble serum glycoprotein composed of a 115-kDa α-chain linked to a 75-kDa β-chain through a single disulfide bond and noncovalent forces. Activation of all three pathways of complement activation leads to the cleavage of C3 into C3b and C3a. It is believed that this cleavage causes a conformational change in the molecule, leading to exposure of a highly reactive thioester bond on C3b, which then allows this molecule to covalently attach to the activating surfaces by forming ester or amide linkages. Further cleavage of C3b, depending on the interacting proteins, yields diverse C3 fragments that interact with six different surface receptors and serve as modulators of important innate as well as adaptive immune functions (Figure1) (Lambris, 1990). One such receptor is complement receptor 2 (CR2, CD21), a 145-kDa type I transmembrane glycoprotein that is expressed primarily on late pre- and mature B lymphocytes, epithelial cells, thymocytes, and follicular dendritic cells. This receptor is composed of an ectodomain, which is comprised of 15 or 16 short consensus repeats (SCRs) or complement control protein (CCP) domains, depending on the splice site usage; a 24-amino acid transmembrane region; and a 34-residue cytoplasmic tail. CR2 plays a role in B-cell activation, the generation of immunologic memory, Ig class switching and B-cell tolerance (Carroll, 1998). These functions are triggered as a result of binding of the receptor to its natural ligands (C3d or iC3b), attached through their thioester bond to an antigen, immune complex or pathogen as a consequence of complement activation. CR2 also serves as a receptor for the Epstein-Barr virus (EBV) surface glycoprotein gp350/220, which through this binding mediates EBV infection of CR2-expressing cells (Nemerow *et al*., 1987; Tanner *et al*., 1987).

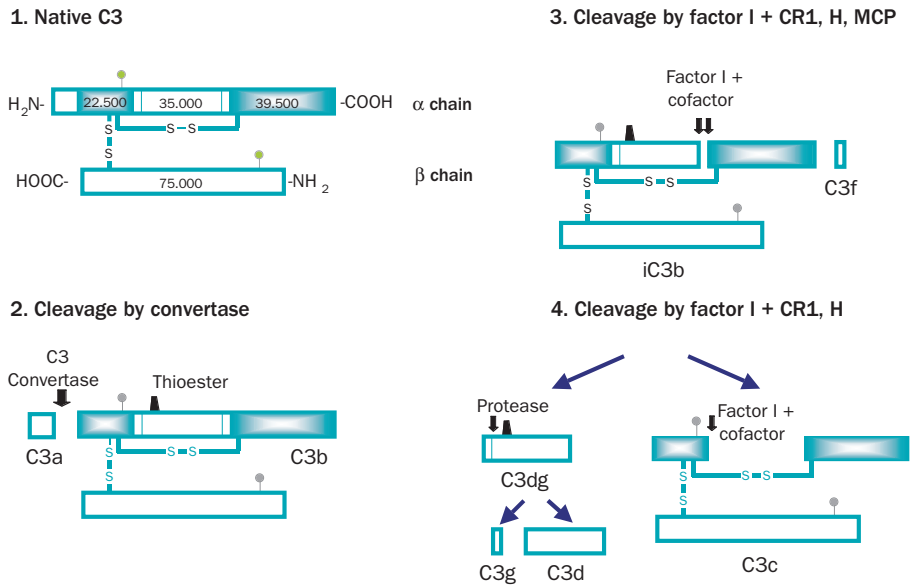

Figure 1. Diagram showing the C3 molecule and the generation of C3 fragments upon complement activation.

Previous studies of the localization of the C3 binding sites for CR2 have yielded apparently contradictory results (Lambris *et al.*, 1985; Esparza *et al.*, 1991; Clemenza and Isenman, 2000; Diefenbach and Isenman, 1995; Kalli *et al.*, 1991; Molina *et al.*, 1991); it is not clear whether the interaction between CR2 and C3 is mediated only through the C3d region, or whether other regions of C3 are also involved in the interaction. In addition, it is not clear whether C3d interacts with CR2 through a single or multiple sites. We speculated that the diverse experimental settings used in the past may have affected these interactions. Key issues that have not been taken into account in studies on the C3d/iC3b-CR2 interaction are that;

- CR2 may have different affinities for soluble and surface-bound C3 molecules (i.e., those bound to the activating particle through their thioester site), since differential expression of various epitopes has already been observed; several monoclonal antibodies vary in their reactivity with the α-chain of C3, depending on whether the C3 fragments are in the fluid phase or are attached to a surface (Nilsson *et al.*, 1992; Aguado *et al.*, 1985). In these experiments, monoclonal antibody 130, which recognizes a neoantigenic determinant that is expressed when C3b is cleaved to iC3b and inhibits CR2 binding, was found to bind C3d more efficiently than iC3b.
- Studies have been performed using the iC3b molecule to identify the C3d residues that interact with CR2, despite the fact that several residues in the C3d region of iC3b may be less accessible to CR2 than those of C3d.

In order to revisit the interactions between CR2 and its natural ligands C3d and iC3b and the EBV surface glycoprotein gp350, we chose to use Biacore's surface plasmon resonance (SPR) technology (Sarrias *et al.*, 2001). Under physiological conditions, C3 fragments are attached to activating particles via thioester linkages, and CR2 is anchored to the cell surface. Therefore, to mimic the actual *in vivo* orientation of these proteins, we biotinylated C3 at the Cys^{988} residue that participates in thioester bond formation and subsequently cleaved the labeled C3 into C3d and iC3b fragments. We then expressed CR2 with a biotinylation tag at its C-terminal end; this tag consisted of amino acids 70-156 of the biotin carboxyl carrier protein (BCCP), a subunit of acetyl CoA carboxylase of *E. coli*, which acts as an acceptor of a biotin molecule in a reaction catalyzed by the biotin holoenzyme synthetase (BirA) protein (Figure 2). These biotinylated proteins were then

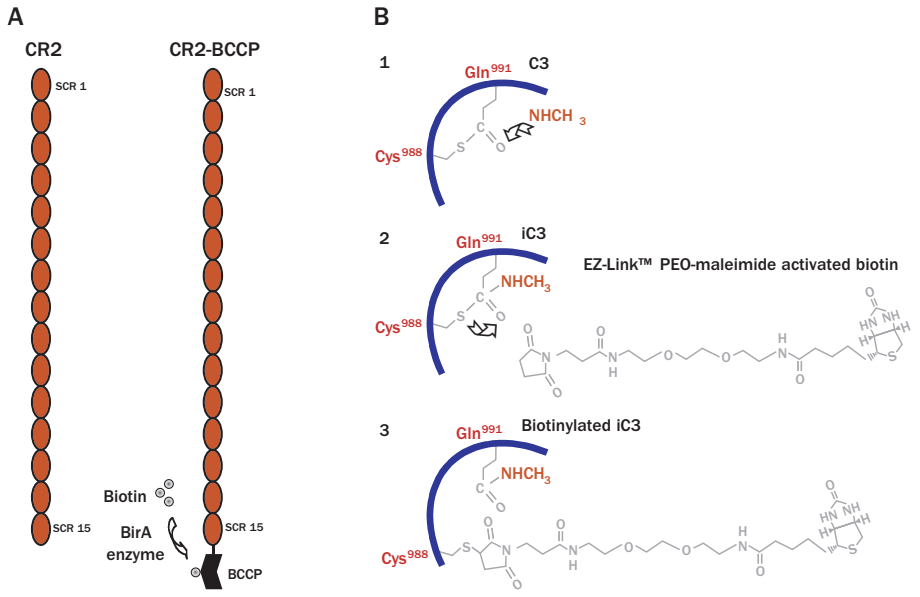

Figure 2. Diagram showing the strategies used to biotinylate CR2 and the C3 fragments at specific sites. CR2 was expressed in fusion with a biotinylation tag in its C terminus (A). Biotin was incorporated to the thioester site of C3 and the molecule was subsequently cleaved to yield the fragments iC3b and C3d (B).

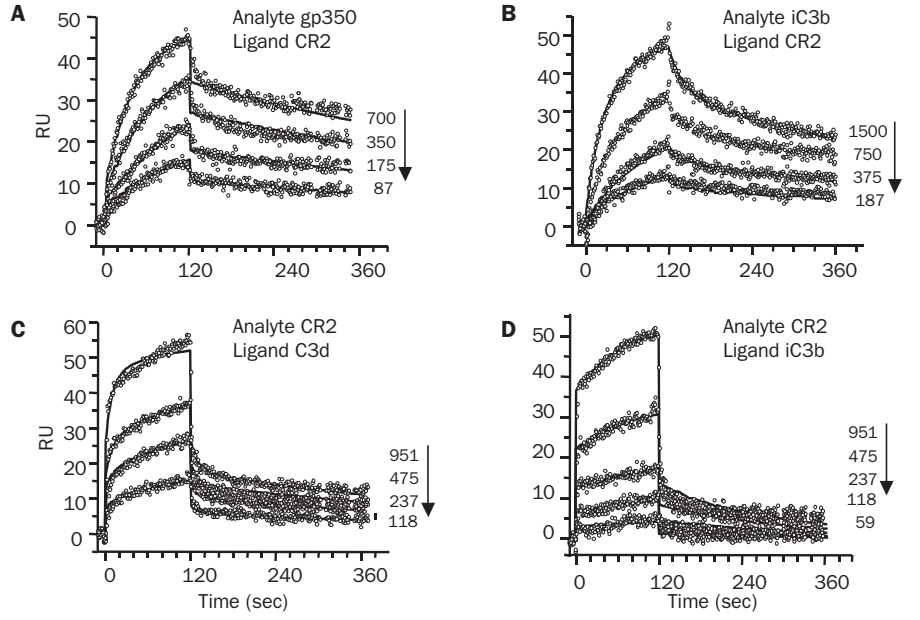

Figure 3. Analysis of the interaction of CR2 with its ligands gp350, C3d and iC3b by SPR. Sensorgram overlays for the binding of gp350 (470t) (A) and iC3b (B) with immobilized CR2-BCCP, and that of soluble CR2 with immobilized C3d (C) and iC3b (D). All the experiments were performed using Biacore X at 25°C in phosphate buffer containing 0.05% Tween 20 (pH 7.4). 140 RU of CR2-BCCP, 250 RU of C3d, or 350 RU of iC3b were immobilized on Fc2 of Sensor Chip SA. Fc1 was used as a blank control. Binding was measured using a rapid flow rate of 50 µl/minute to minimize mass transport limitation. Flow was allowed to occur for several seconds to establish a baseline and then various concentrations of analyte (nM) were injected (indicated at the right hand side of the sensorgram). The association was allowed to proceed for 120 seconds, at which time sample was replaced with running buffer and the dissociation of the complex was monitored for 240 seconds. BIAevaluation 3.0 software was used to analyze the binding data, using global fitting analysis. Solid lines are the result of the global fitting analysis. gp350 (470t) binding to CR2 (A) fit a 1:1 langmuir model, whereas binding of the C3 fragments to the receptor fit a bivalent interaction model.

oriented onto Biacore's streptavidin precoated Sensor Chip SA. Orientation of the proteins in this way produced a homogeneous ligand surface that facilitated the measurement of homogeneous binding constants.

Analysis of our binding data showed that binding of the N-terminal region of gp350, gp350 (470t), to CR2 immobilized on the sensor chip followed a simple 1:1 interaction with an affinity dissociation constant (K_D) of 45 nM. These data also excluded the existence of experimental artifacts and validated our experimental design. In contrast to the CR2-gp350 interaction, binding of soluble CR2 to immobilized C3d and iC3b was more complex and involved more than one intramolecular component (Figure 3). Global fitting analysis of the binding data indicated that the interaction between CR2 and C3d/iC3b follows a bivalent model. These data also suggested that multiple regions in C3 interact directly with multiple regions in the receptor.

Previous data from our laboratory and others have indicated that at least two regions of human C3d are involved in its interaction with CR2, i.e., two clusters of negatively charged residues (based on X-ray crystallography studies) (Clemenza and Isenman, 2000) and the region in C3 comprising aa 1199-1210 (based on synthetic peptide binding studies) (Esperanza *et al.*, 1991). However, when the structure of the C3d-CR2 (SCR1-2) complex became known, it was shown that the association site was neither at, nor close to the acidic pocket and did not involve the region spanning residues 1199-1210 (Szakonyi *et al.*, 2001). This is contradictory to mutagenesis data provoking an intuitive conclusion that residues located at the association interface are solely responsible for association. The mechanism of C3d-CR2 association, therefore, has been a puzzle up to now. Based on the calculation of electrostatic potentials for the free and bound species and apparent pKa values for each ionizable residue, it has recently been shown that association between C3d and CR2 is electrostatic in nature and involves not only the association interface, but both molecules in their entirety (Morikis and Lambris, 2004). To reassess the involvement of the C3d region spanning residues 1199-1210 in the interactions with

the whole CR2 (SCR 1-15), we performed a direct binding assay using Biacore's SPR technology.

We observed direct binding of CR2 to a synthetic peptide with a sequence comprising C3 amino acids K1187-A1212 (the peptide was biotinylated at the N-terminus and oriented on the streptavidin-coated chip), suggesting that this region in C3d is involved in its interaction with the receptor. These binding data allowed us to observe that the dissociation of CR2 from C31187-1214 appeared to occur much faster than that from C3d. This result suggested that regions of C3d other than the charged clusters might be involved in its interaction with CR2, possibly stabilizing the complex.

Although both CR2-C3d and CR2-iC3b interactions were complex, we found kinetic differences between the two. We hypothesized that these differences may be related to the participation of CR2-interacting residues in the C3c fragment. In fact, CR2 was found to bind to C3c when the latter was immobilized on a sensor chip by amine coupling chemistry. It is particularly interesting that in our study we also found that the orientation of the molecules in the CR2-iC3b interaction is important. Biacore analysis showed that iC3b binds to CR2 with different kinetics depending on whether the iC3b is in solution or immobilized on the surface. These results suggested that CR2 may have different affinities for soluble and surface-bound C3 fragments *in vivo*, or that immobilization affects one of the two ligands.

In summary, we have used Biacore to gain insight into the interaction of CR2 with its ligands. Our results indicated that the binding of CR2 to viral gp350 follows a simple 1:1 binding model, whereas its binding to C3 fragments is more complex than previously thought. We observed kinetic differences between the binding of iC3b and C3d to CR2, a finding which suggested that the regions of iC3b and C3d involved in the interaction with CR2 may, in addition to the common contact sites, also involve regions that differ for the two molecules.

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