Brief Definitive Report

FUNCTIONAL AND ANTIGENIC PROPERTIES OF COMPLEMENT RECEPTOR TYPE 2, CR2

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C3d receptor (CR2) was first described as a receptor for C3d, distinct from the C3b receptor (CR1), expressed by B lymphocytes (1, 2), and identified as a glycoprotein of 140,000 M_r with an mAb anti-B2 (3). This same molecule serves as the receptor for Epstein-Barr virus (4). Involvement of CR2 in a pathway of B-lymphocyte activation has been suggested from studies using anti-CR2 (5, 6) and multivalent C3d (7).

CR1, C4-binding protein (C4-bp), and factor H are encoded by tightly linked genes (8), and cDNA sequencing studies show (9–11) that each protein contains consensus repeating homology units of ~60 amino acids. Moreover, they have certain similarities in their function: accelerating decay of the C3 convertases and serving as cofactors of C3b inactivator (I). Thus, it has been proposed that they are part of a gene family of C3- and C4-regulatory proteins.

Weis et al. (12) identified a partial cDNA clone for CR2 that crosshybridized with CR1 cDNA probe, and showed a strong homology in tryptic peptide sequences of CR2 and CR1. Based on these findings, they postulated CR2 being a member of the gene-family, although no regulatory activity shared with other members had yet been shown.

In this report, we will demonstrate that CR2 has functional activity, serving as a cofactor of I for the cleavage of membrane-bound iC3b into C3dg and C3c, and that it shares antigenicity with CR1.

Materials and Methods

Antibodies. Hybridoma cell line HB-5 secreting IgG2a monoclonal anti-CR2 was obtained from American Type Culture Collection, Rockville, MD. OKB7, a IgG2a monoclonal anti-CR2 was purchased from Ortho Diagnostic Systems Inc. (Westwood, MA). mAbs 57H and 44D were against human CR1. As controls, other mAbs were used: TK1, a IgG1κ monoclonal anti-C4-bp; and 103A, a IgG2aκ monoclonal anti–human monocyte.

Complement Components and Intermediate Cells. Sheep erythrocytes sensitized with rabbit hemolysin (EA) were reacted with large excess of guinea pig C1 and oxidized human C2 and 30 site-forming units (SFU) of human C4, and then with ^{125}I-labeled C3. They were converted to EAC14oxy23bi by incubation with I (2 μg/ml) and H (6 μg/ml) at 37°C for 1 h, and referred to as EA^{125}I-iC3b.

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Immunoelectroblotting Analysis. After SDS-PAGE, samples were electrically transferred to Hydrophobic Durapore sheets (Millipore Limited Co., Tokyo, Japan). The sheets were soaked in 1:2 dilution of PBS with distilled water (0.5 × PBS) containing 5% BSA for 30 min at room temperature, and then they were incubated with first antibody in 1% BSA and 0.5 × PBS containing 1% goat serum for 1 h. After washing with 0.05% Tween 20 in 0.5 × PBS, the sheets were treated with Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA).

Raji Cell Culture and Preparation of the Cell Lysate. About 10^{10} Raji cells were washed twice with PBS and solubilized in 1 liter of 10 mM Tris-HCl buffer, pH 8.3, containing 0.55% NP-40, 5 mM (*p*-Amidinophenyl) methanesulfonyl fluoride hydrochloride (*p*-APMSF), 20 μg/ml soybean trypsin inhibitor, 100 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 50 U/ml Trasilol with continuous stirring for 2 h in an ice bath. Nuclei and other insoluble material were removed by centrifugation.

Purification of CR2. The detail of the first chromatography on DEAE-Toyopearl (Toyosoda Co.) is described in the legend of Fig. 1. CR2 pool was dialyzed and subjected to the second ion exchange chromatography on SP-Toyopearl 650S (1.5 × 15 cm) preequilibrated with 10 mM phosphate buffer, pH 6.2, containing 10 mM NaCl, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) and inhibitors, 10 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 5 mM *p*-APMSF. CR2 was eluted with salt gradient at an NaCl concentration between 0.2 and 0.3 M. For further purification, a CR2-enriched pool was applied to sequential columns of Sepharose–mouse IgG and Sepharose-HB-5 at 6 ml/h at 4°C. The Sepharose-HB-5 column was washed successively with 30 ml of 150 mM NaCl/20 mM Tris-HCl, pH 8.3, containing 0.1% Chaps and the inhibitors, with 30 ml of 150 mM NaCl/50 mM acetate, pH 4.5, containing the same detergent and inhibitors, and finally with 30 ml of the same Tris buffer used in the first wash. CR2 was eluted with 3M potassium thiocyanate containing 0.1% Chaps and the inhibitors, followed by dialysis with PBS containing 0.1% Chaps and then it was concentrated to 0.5 ml by centrifugation at 100,000 *g* for 2 h. Protein concentration of the final CR2 preparation was determined by the method of Lowry using BSA as a standard.

Results

CR2 was purified from NP-40 lysate of Raji cells. Fig. 1 *a* shows the elution profile from DEAE-Toyopearl 650S. Degradation products of CR2 with various sizes (120, 100, 17, 14, and 11 × 10^3) were observed. After the second ion exchange chromatography on SP-Toyopearl 650S, pooled fractions were completely free of degraded CR2 fragments. Since a small amount of CR1 was detected in the Raji cell lysate by immunoelectroblotting, the CR2 preparation was treated with 100 μl of Sepharose-anti-CR1 (57H + 44D) for 4 h at 4°C. Finally, 120 μg of CR2 was obtained, and it was shown as a single Coomassie blue–stained band on SDS-PAGE (Fig. 1 *b*).

Purified CR2 was examined for its cofactor activity in the I-mediated cleavage of membrane-bound iC3b. EA^{125}I-iC3b were incubated with purified CR2, washed, and then incubated with I. Substantial amounts of C3 radioactivity (7.7%) were released upon incubation with buffer, I alone, or CR2 alone. 13.9% of the radioactivity were released from the cells treated with CR2 plus I, and 17.0% from cells treated with CR1 plus I. The structural analysis on SDS-PAGE indicated that iC3b, 75-kD chain and 65-kD chain, was released from the controls. An additional band of 31 kD was observed in the supernatants from cells incubated with CR2 plus I or CR1 plus I under reducing conditions. And an additional band of 140 kD was observed under nonreducing conditions, indicating that C3c was released. As already described, the 40 kD peptide was poorly labeled (13). The release of C3c was inhibited when CR2 was pretreated with

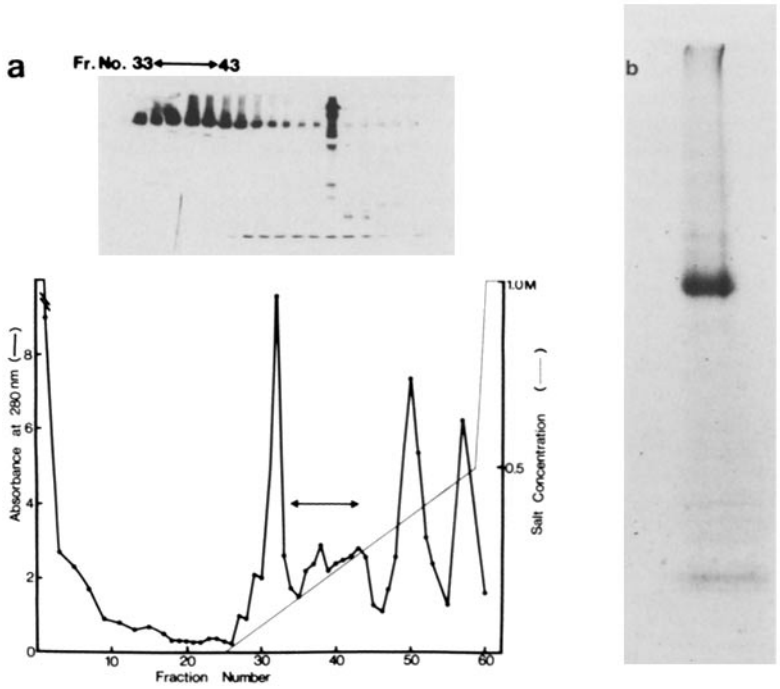

FIGURE 1. Purification of CR2. 1 liter of the lysate was applied to a DEAE-Toyopearl column (1.5 × 15 cm) preequilibrated with 10 mM Tris-HCl buffer, pH 8.3, containing 10 mM NaCl and 0.5% NP-40. The column was washed with Tris-HCl buffer containing 10 mM NaCl, 0.1% Chaps, 10 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 5 mM *p*-APMSF and was eluted with a linear salt gradient at a flow rate of 3 ml/min. 10-ml fractions were collected and assayed for CR2 by immmunoelectroblotting. The inset (*a*) shows the results of the assay, indicating intact CR2 and degraded molecules were detected with HB-5. (*a*) Fractions 34–43 were pooled. (*b*) 1.2 μg of purified CR2 was run on a 5–15% SDS-PAGE gradient gel under reducing conditions and stained with Coomassie blue.

OKB7 anti-CR2, whereas no inhibition was observed with 103A control IgG2a mAb. However, when CR2 was preincubated with 57H anti-CR1, partial inhibition of C3c release was noticed (Fig. 2). These results raised two possibilities: (*a*) that CR2 crossreacted with 57H anti-CR1 and (*b*) that a small amount of CR1 was present in the CR2 preparation. To exclude the latter possibility, the amount of possibly contaminating CR1 in the CR2 preparation was measured by semiquantitative immunoelectroblotting where serially diluted CR1 and CR2 were tested. The 140 kD band was the only band detected in the CR2 preparation with 57H, even when 1.2 μg of CR2 was loaded; in this assay 0.6 ng of CR1 could be detected. To further confirm that CR2 but not CR1 did serve as a cofactor of I, we tested whether CR2 could also act on membrane-bound C3b as a cofactor of I. Three times more CR2 was necessary to achieve the similar extent of C3c release from C3b (data not shown). On the contrary, CR1 was more effective in serving as a cofactor of I for cleaving C3b than iC3b, as previously reported (13).

Next we examined the possibility that CR2 shares the antigenicity with CR1 by immunoelectroblotting. 57H is an mAb against CR1 that recognizes an epitope

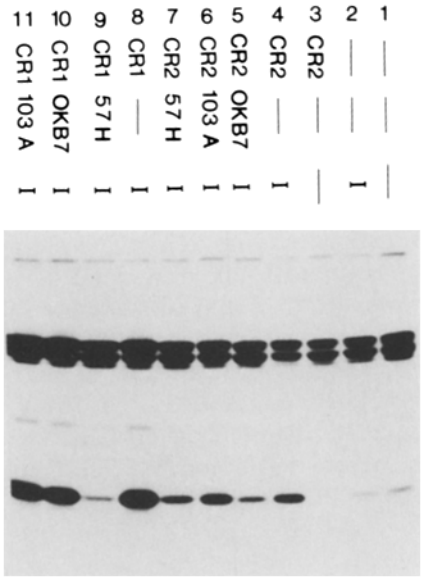

FIGURE 2. SDS-PAGE analysis of the cleavage products released from EA^{125}I-iC3b. CR2 (200 ng), CR1 (2.5 ng), or buffer were incubated with either antibody (400 ng), OKB7, 57H, or 103A at 37°C for 30 min before addition to EA^{125}I-iC3b (4×10^6 cells) in a total volume of 80 μl. After a 30-min incubation with CR2, CR1, or buffer, cells were washed twice and resuspended in 80 μl of the buffer, and then 20 μl of I (200 ng) or buffer were added. After a 30-min incubation at 37°C, cells were centrifuged and the supernatants were analyzed by SDS-PAGE 10% gel under reducing conditions followed by radioautography. Isotonic veronal-buffered saline containing 2.5% dextrose, 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, supplemented with 0.1 mg/ml BSA was used in the assay.

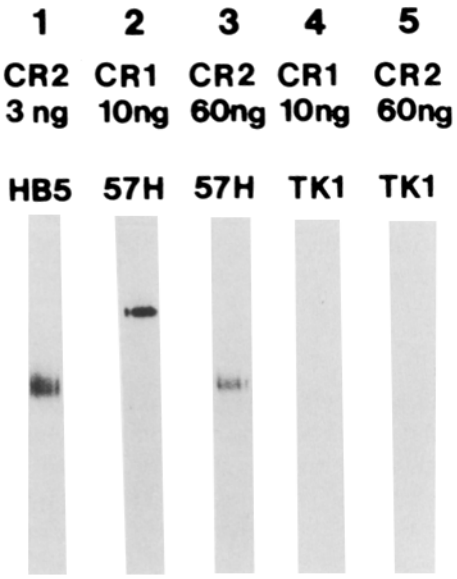

FIGURE 3. Crossreactivity of 57H anti-CR1 with CR2. Purified CR1 and CR2 were run on 6.5% SDS-PAGE and analyzed by immunoblotting as follows: (*1*) CR2 (3 ng) detected with HB-5; (*2*) CR1 (10 ng) detected with 57H; (*3*) CR2 (60 ng) detected with 57H; (*4*) CR1 (10 ng) detected with TK1; (*5*) CR2 (60 ng) detected with TK1.

at or in the close proximity of the ligand binding site of the receptor. When a fairly large amount (60 ng) of CR2 was used, it reacted with 57H but not with a control mAb TK1 anti-C4bp (Fig. 3). Crossreactivity of OKB7 with CR1, however, was not detected.

Discussion

We investigated the functional and antigenic properties of CR2 in its purified form. No functional activity of CR2 in regulation of complement cascade has yet been shown. If CR2 has any regulatory function on C3, it can be expected to act

on membrane-bound iC3b. This assumption was made because of the following reasons. iC3b is recognized by CR2 as a better ligand than C3b with far higher avidity (14), and moreover, the ligands of complement receptors are well recognized when they are in clusters on immune complex or membrane. CR2 indeed exhibited a cofactor activity of I in cleaving bound iC3b into C3dg and C3c. A previous study by Weis et al. (15) showed that CR2 lacked functional activities; it failed to accelerate the decay of C3 convertases and to serve as a cofactor of I in cleaving fluid phase C3b. Therefore, this function, serving as a cofactor for cleavage of bound iC3b by I, may be the only regulatory function of CR2 on complement cascade. It could be argued, however, that a small amount of contaminating CR1 in the CR2 preparation was responsible for the cofactor activity. Several lines of evidence excluded this possibility. First, the contaminating CR1 was estimated as <0.05% by a semiquantitative immunoelectroblotting. Second, the cofactor activity of CR1 was completely inhibited when CR1 was preincubated with 57H anti-CR1, while only partial inhibition by 57H was observed for the cleavage with CR2 plus I (Fig. 2). Third, CR2 was more effective on EAiC3b than on EAC3b, contrary to the effect of CR1. Thus it is clear that CR2 is responsible for the cofactor activity.

Another important finding is that CR2 crossreacted with 57H monoclonal anti-CR1 (Fig. 3). The crossreaction was indicated also in the functional assay where 57H inhibited the CR2 plus I–mediated C3c release (Fig. 2). These results suggest that CR2 and CR1 have similar structures in the site that is involved in the regulatory function.

Taken together, our findings that CR2 has regulatory function on iC3b and that CR2 crossreacts with anti-CR1 imply that CR2 and CR1 may be evolutionally related. The physiological role of the cofactor function of CR2 has to be determined by further study since the activity was very weak, ~120–400 times less effective than CR1 on a weight basis. However, these observations will provide further evidence to place CR2 as a new member of the gene family of C3- and C4-regulatory proteins.

Summary

This is the first report demonstrating that C3d receptor (CR2) has functional activity in regulating complement cascade. Purified CR2 was examined for its cofactor activity in factor I–mediated cleavage of membrane-bound iC3b. CR2 plus C3b inactivator (I) released C3c from EA^{125}I-iC3b, and the release was inhibited when CR2 was preincubated with OKB7 monoclonal anti-CR2. Furthermore, immunoelectroblotting analysis showed crossreactivity of CR2 with 57H anti-CR1. These results indicate that CR2 has functional and antigenic similarity to CR1, thus providing a supporting evidence for placement of CR2 as a member of the recently defined gene family of C3- and C4-regulatory proteins composed of CR1, C4-binding protein, and factor H.

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