Distinct Receptor and Regulatory Properties of Recombinant Mouse Complement Receptor 1 (CR1) and Crry, the Two Genetic Homologues of Human CR1

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Summary

The relationship between the characterized mouse regulators of complement activation (RCA) genes and the 190-kD mouse complement receptor 1 (MCR1), 155-kD mouse complement receptor 2 (MCR2), and mouse p65 is unclear. One mouse RCA gene, designated *MCR2* (or *Cr2*), encodes alternatively spliced 21 and 15 short consensus repeat (SCR)–containing transcripts that crosshybridize with cDNAs of both human *CR2* and *CR1*, or *CR2* alone, respectively. A five SCR–containing transcript derived from a second unique gene, designated *Crry*, also crosshybridizes with human *CR1*. We have previously shown that the 155-kD MCR2 is encoded by the 15 SCR–containing transcript. To analyze the protein products of the other transcripts, which are considered the genetic homologues of human *CR1*, we have expressed the 21 and the 5 SCR–containing cDNAs in the human K562 erythroleukemia cell line. We demonstrate that cells expressing the 21 SCR transcript express the 190-kD MCR1 protein. These cells react with five unique rat anti-MCR1 monoclonal antibodies, including the 8C12 antibody considered to be monospecific for MCR1. In addition, these cells efficiently form rosettes with mouse C3b-bearing sheep erythrocytes. In contrast, cells expressing the five SCR–containing *Crry* transcript are strongly recognized by an anti-human CR1 antibody that also defines the mouse p65 protein. Using a functional assay that measures the surface deposition of C3 activated via the classical complement pathway, we show that Crry/p65-expressing cells have a markedly decreased amount of C3 deposited on them as compared with control cells expressing the antisense construct or cells expressing MCR1 or MCR2. This suggests that Crry has intrinsic complement regulatory activity. Overall, these studies demonstrate that mouse has an RCA gene family that encodes proteins with similar activities and biologic roles, as compared with their human counterparts, but with very unique structures.

The complement system protects an individual against foreign organisms and antigens by promoting chemotaxis and phagocytosis, increasing the lysis and clearance of pathogens, and participating in the trafficking of immune complexes through the circulation (reviewed in reference 1). While this system is highly effective, it has also been recognized that activated complement fragments have the capacity to bind and attack self tissues, especially in areas of active inflammation. To protect autologous bystander cells from the deleterious effects of complement, several membrane-bound and serum proteins have evolved in humans. These proteins interact with the complement cascade and, by inactivating its convertase enzymes, are believed to protect self tissue (reviewed in references 2–6).

Four complement receptor and regulatory proteins are present on the surface of human cells and interact with complement component C3, an essential participant in this system. These proteins are encoded by the regulators of complement activation (RCA)[1] gene family located on the long arm of chromosome 1 (7–9). Included in this RCA family is human

[1] *Abbreviations used in this paper:* DAF, decay-accelerating factor; MCP, membrane cofactor protein; RCA, regulators of complement activation; SCR, short consensus repeat.

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CR1, a 190-kD glycoprotein that binds C3b-coated targets. CR1 also acts as a cofactor for factor I–mediated C3b and C3bi cleavage and subsequently inactivates the convertase containing this molecule. Human CR1 also accelerates decay of the C3 convertases. Membrane cofactor protein (MCP), another family member, is an integral membrane glycoprotein found on a wide variety of cells that also has cofactor activity for factor I–mediated C3b cleavage. Decay-accelerating factor (DAF), a third member, is a glycolipid-anchored protein that both inhibits the formation of and accelerates the decay of the C3 convertases. The fourth member, human CR2, has been reported to act as a cofactor for factor I–mediated cleavage of C3bi in addition to serve as a receptor for C3d,g and the EBV.

Several investigators have analyzed mouse counterparts of the human RCA family using ligand binding, immunochemical, and molecular hybridization techniques. Three proteins that bind mouse C3-Sepharose have been identified on the membrane of murine splenocytes (10, 11). The largest, a 190-kD protein designated mouse CR1 (MCR1), has receptor activities and mediates rosette formation with mouse C3b-coated sheep erythrocytes. In addition, MCR1 demonstrates regulatory functions that resemble human CR1 in that it serves as a fluid phase cofactor for factor I–mediated cleavage of mouse C3b and C3bi (10).

The second largest C3-Sepharose binding protein, 155 kD, is believed to be the mouse homologue of human CR2. This protein, designated MCR2 (or Cr2), mediates rosette formation with mouse C3d-coated erythrocytes (12) and is encoded by a gene within the mouse RCA locus (13–16) that has extensive similarity to the human *CR2* gene (16). This conclusion is substantiated by the observation that expression of a 15 short consensus repeat (SCR)–containing cDNA derived from this gene results in a protein with identical M_r and activities as compared with the 155-kD splenic MCR2 protein (17). Of interest, four of five mAbs directed against the 190-kD MCR1 protein also react with the 155-kD MCR2 (10–12, 16, 17). A 21 SCR-containing alternatively spliced transcript has also been found to be derived from the *MCR2* gene, and the 190-kD MCR1 has been proposed to arise from this transcript (14). However, this has not been previously formally demonstrated.

Recently, a 60-kD membrane protein with DAF-like properties has been isolated from mouse erythrocytes; however, the sequence of its protein and gene, in addition to its capacity to bind C3-Sepharose, is unknown (18). No genetic DAF or MCP homologues have been yet found in mice despite attempted identification by cross-species hybridization techniques (15; V. M. Holers, unpublished observations; and J. P. Atkinson, personal communication).

A third mouse spleen cell guinea pig C3b-Sepharose binding protein, p65, has been characterized due to its ability to cross-react with a polyclonal rabbit anti–human CR1 antiserum (19). p65 is widely distributed, present on murine splenocytes, lymph node cells, peritoneal macrophages, erythrocytes, and L929 cells. Although p65 binds to guinea pig C3b-Sepharose, the physiologic function of p65 has been undefined. In addition, its relationship to p60, the third mouse C3-Sepharose binding protein (10), and *Crry*, a human *CR1* genetic homologue, has been unclear. The *Crry* gene was identified in mice by hybridization studies using human *CR1* probes against mouse genomic and cDNA libraries (20–23). *Crry* has been reported to encode a 70-kD protein consisting of a signal peptide, five SCRs, a transmembrane, and intracytoplasmic domain. Although similar to areas of human CR1 in its sequence, the broad tissue distribution of *Crry* transcripts has suggested that the Crry protein does not have comparable C3 receptor functions. The function of the *Crry* gene product, or indeed its relationship to the complement pathway, has been unknown.

To further understand the interelationships between the mouse genetic and functional homologues of human RCA proteins, we have undertaken to express and analyze recombinant forms of each homologous mouse cDNA. Herein we report our analysis of recombinant *MCR1* and recombinant *Crry*, both of which contain sequences that hybridize to human *CR1* cDNA probes and are, therefore, considered to be genetic homologues of human *CR1*. We demonstrate that the 190-kD MCR1 is encoded by the 21 SCR–containing alternatively spliced transcript arising from the *MCR2* (*Cr2*) gene and that the previously described protein, p65, is encoded by the *Crry* gene. In addition, we show that cells expressing the Crry/p65 protein are remarkably protected from classical pathway-mediated complement attack as measured by a significant decrease in mouse C3 deposition on their surface. This intrinsic regulatory activity is in contrast to cells expressing either MCR1 or MCR2. Overall, these studies show that Crry/p65 has important complement regulatory activities and that in mice, a unique set of proteins has evolved that act as C3 receptors and that protect self tissue from complement-mediated damage.

Materials and Methods

Cloning and Expression of MCR1 and Crry cDNAs. By primer extension and PCR-based methods using RNA from BALB/c splenocytes, we obtained a cDNA containing the initial six SCRs of the previously described 21 SCR–containing transcript encoded by the *MCR2* gene (14). This partial cDNA was cloned into the 5′ portion of our 15 SCR–containing plasmid pBSMCR2-5 (16, 17) to obtain the full-length 21 SCR–containing cDNA. To express the *Crry* transcript, we used a cDNA that we had previously isolated (23) from a BALB/c peritoneal macrophage cDNA library cloned into λgt10 and screened with a partial human *CR1* cDNA (24). This clone was sequenced and shown to be essentially identical to the nonintervening sequence containing *Crry* cDNA (20). An EcoRI site near the 3′ end was altered by PCR-based mutagenesis without changing the predicted protein sequence. Both cDNAs were cloned into the pSFFV-neo plasmid in sense or antisense orientations and transfected by electroporation into the human K562 erythroleukemia cell line using previously described techniques (25).

Preparation and Characterization of Antibodies. Anti-K562 polyclonal antibody was prepared by emulsification of 10^8 wild-type K562 cells with Freund's adjuvant followed by serial immunization of New Zealand rabbits. The immune antiserum was assayed by flow cytometry and rabbit complement–mediated cellular cytotoxicity to assure its anti-K562 reactivity. Immunoglobulins from the reactive antiserum were isolated by ammonium sulfate precipi-

tation. The affinity-purified rabbit $F(ab')_2$ anti-human CR1/p65 antiserum was the same reagent used in previous studies (19). The rabbit polyclonal anti-human DAF antiserum and the murine mAb to human MCP, TRA-2-10(26, 27), were generously provided by T. Oglesby and J.P. Atkinson (Washington University School of Medicine, St. Louis, MO). 7E9, 7G6, 8C12, 4E3, and 8D9 are rat anti-MCR1 mAbs (11, 12), and BRN-1 is a rabbit polyclonal antiserum raised against a fusion protein expressing 10 SCRs of MCR2 (16).

Flow Cytometric Analysis. 10^6 K562 cells expressing recombinant Crry (rCrry) in sense or antisense orientations were incubated at 4°C in 25 μl of HBSS containing 1% human serum albumin with 120 μg/ml of nonimmune rabbit $F(ab')_2$ (Cappel Laboratories, West Chester, PA) or affinity-purified rabbit $F(ab')_2$ anti-human CR1/p65 as a first-step antibody. After 60 min, cells were washed, and FITC-conjugated goat $F(ab')_2$ anti-rabbit $F(ab')_2$ (Cappel Laboratories) was added. After a 60-min incubation and washes, cells were analyzed by flow cytometry on a FACScan® (Becton Dickinson & Co., Palo Alto, CA).

Analysis of DAF and MCP expression was performed by using 10^6 cells in a buffer containing PBS, 1.0% BSA, and 0.02% NaN_3. First-step antibodies included equal amounts of either nonimmune rabbit serum or immune rabbit anti-DAF serum, and 7 μg each of MOPC-21 (Sigma Chemical Co., St. Louis, MO) or anti-MCP mAb. FITC-conjugated goat $F(ab')_2$ anti-rabbit Ig (Cappel Laboratories) or anti-mouse Ig (Cappel Laboratories) were used as second-step antibodies. Analysis of recombinant MCR1 (rMCR1) and MCR2 (rMCR2) expression was performed by using 1 μg of the rat anti-MCR1 mAb. FITC-conjugated goat anti-rat Ig (Cappel Laboratories) was used as the second-step antibody.

Immunoprecipitation and SDS-PAGE Analysis. K562 cells expressing rMCR1 and BALB/c splenocytes were surface labeled with ^{125}I and solubilized using PBS containing 1% NP-40 and protease inhibitors as previously described (16). Solubilized cell preparations were incubated either with preimmune or immune BRN-1 serum. The immune complexes were collected using protein A–Sepharose (Pharmacia, Uppsala, Sweden) and washed four times with PBS/1% NP-40. Pellets were treated with SDS-PAGE sample buffer and heated to 85°C for 5 min. Eluted samples were analyzed by 7.5% SDS-PAGE.

Erythrocyte Rosette Formation. Sheep erythrocytes (E) bearing complement components were prepared as previously described to obtain reagents bearing forms of either human (28) or mouse (12) C3. For analysis, 1.5×10^5 K562 cells expressing sense orientation rMCR1, sense orientation rMCR2, sense or antisense orientation rCrry, or wild-type K562 cells in 100 μl PBS were incubated in 50 μl of HBSS with 1% human serum albumin, 2 mM $MgCl_2$, and 0.15 mM $CaCl_2$. The cells and Es were gently mixed, centrifuged at 500 rpm for two min at 4°C, and incubated for 20 min at 37°C. Cells with three or more adherent Es were scored as positive. To test the effect of mAbs on E-rosette formation, cells were preincubated with 10 μg/ml of antibody for 15 min at room temperature before E addition.

Detection of C3 Deposition. 10^6 cells in 100 μl of RPMI containing 2 mM $MgCl_2$ and 0.15 mM $CaCl_2$, or 2 mM $MgCl_2$ and 10 mM EGTA, were incubated with 100 μg of purified rabbit anti-K562 polyclonal antibody and 20 μl (see Figs. 4 and 5) or increasing amounts (see Fig. 6) of BALB/c mouse serum, or equal amounts of heat inactivated BALB/c mouse serum obtained at the same bleeding. Incubation was performed at 37°C for 60 min. After washing with PBS containing 1.0% BSA, the cells were analyzed for the presence of mouse C3 on their surface by a variation of a previously described technique (29). FITC-conjugated goat anti-mouse C3 antibody (Cappel Laboratories) was added at 4°C for 30 min followed by washing and flow cytometry as above. Heat inactivation of the murine serum was performed at 56°C for 60 min. In preliminary experiments using ^{51}Cr-labeled K562 cells, all of these conditions were shown to be noncytotoxic.

Results

Expression of rCrry. Nucleotide sequence analysis of our *Crry* cDNA revealed it to be almost identical to the sequence of Paul et al. (21), containing a signal peptide, five SCRs, a transmembrane, and an intracytoplasmic region. Slight difference noted were the presence of a GC instead of a CG at nucleotides 537 and 538 (21), changing a single amino acid from alanine to glycine in the area of the signal peptide, and a C instead of a G at nucleotide 965, changing a glutamic acid to glutamine within the first intercysteine region of the second SCR. We subcloned this cDNA into the eukaryotic expression vector pSFFV-neo and stably transfected it into the human K562 erythroleukemia cell line.

Although the identity of the protein encoded by the *Crry* transcript was unknown, we believed that it might be the previously described p65 (19). To test this hypothesis, we analyzed by flow cytometry our rCrry-expressing K562 cells using the affinity purified $F(ab')_2$ anti-human CR1/p65, which specifically recognizes p65 on the surface of murine cells. This antibody does not immunoprecipitate the 190-kD MCR1 or the 155-kD MCR2 proteins. Flow cytometric analysis of stable lines obtained from transfection with the rCrry sense and antisense constructs are shown in Fig. 1. Cells transfected with the sense orientation construct react strongly with the anti-human CR1/p65 antibody, as compared with the antisense transfected control (Fig. 1 *A*). No specific staining of either cell population is detected with the nonimmune rabbit $F(ab')_2$ control (Fig. 1 *B*). Three separate transfections gave identical results. As a further control, no human CR1 is expressed by these transfected cells, as assessed by the lack of staining with the anti-human CR1 mAb 3D9 (data not shown). These results demonstrate that *Crry* encodes the p65 protein.

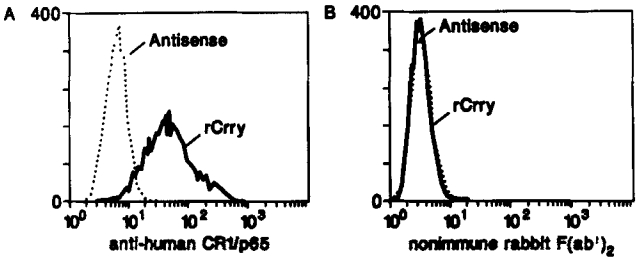

Figure 1. (*A*) Flow cytometric analysis demonstrating expression of p65 by rCrry sense orientation constructs (*solid line*), but not antisense constructs (*dotted line*), in K562 cells as detected by anti-human CR1/p65 antibody. *x*-axis is relative fluorescence on a log scale; *y*-axis is cell number. (*B*) Flow cytometric analysis demonstrating lack of reactivity of cells expressing either rCrry sense (*solid line*) or antisense (*dotted line*) constructs with control antibody.

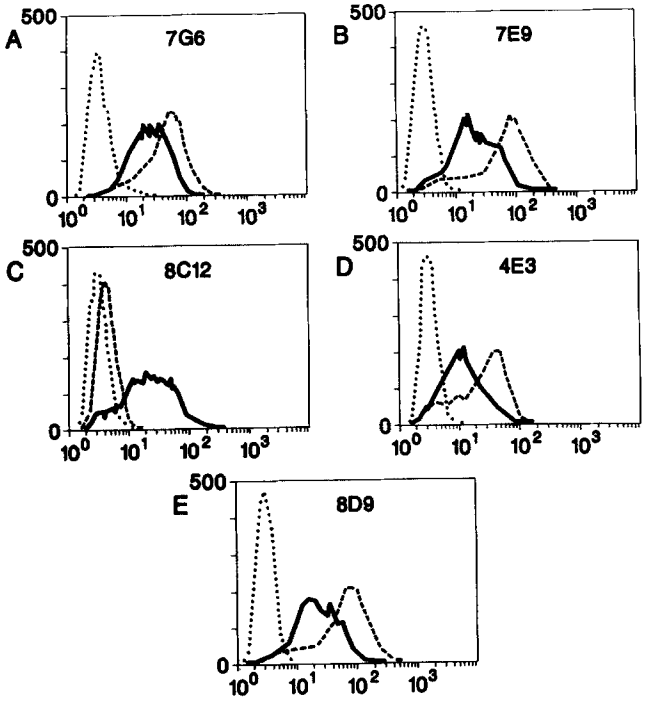

Figure 2. Flow cytometric analysis using five different rat anti-MCR1 antibodies on K562 cells expressing rMCR1 (*solid line*), rMCR2 (*dashed line*), or the rMCR1 antisense construct (*dotted line*). *x*-axis is relative fluorescence on a log scale; *y*-axis is cell number.

Expression of the 21 SCR Containing MCR2 (Cr2) Gene-derived Transcript (rMCR1). The cDNA we cloned and expressed is essentially identical to the alternative 21 SCR–containing transcript derived from the *MCR2* (*Cr2*) gene as described by Kurtz et al. (14). Differences noted are an A instead of a C nucleotide 336, which does not change the amino acid sequence. A second difference is the absence of one complete codon, AGC, beginning at nucleotide 541 of the Kurtz sequence. This triplet encodes a serine in the first intercysteine region in the third SCR of the mature protein. The nature of this difference is currently unknown; however, there appears to be no significant functional or antigenic consequence (see below).

After subcloning this cDNA into pSFFV-neo and expressing it in the K562 cell line, we analyzed its relationship to the 190-kD MCR1 protein. As shown in Fig. 2, flow cytometric analysis reveals that cells transfected with the sense orientation construct are recognized by five rat mAbs raised against MCR1, including the mAb 8C12 (Fig. 2 *C*). Four of these mAbs also react with MCR2; however, 8C12 is monospecific for MCR1. Indeed, cells transfected with rMCR2 are stained by all mAbs except 8C12 (17, and Fig. 2 *C*). Cells transfected with the antisense construct of the 21 SCR containing transcript and wild-type K562 cells are not recognized by any of the mAbs. In addition, the rMCR1- and rMCR2-expressing cells are not stained by the anti-human CR1/p65 antibody to a detectable level by flow cytometry (data not shown).

To further demonstrate that MCR1 is encoded by the 21 SCR–containing transcript, we performed an immunoprecipitation analysis using BRN-1, a rabbit polyclonal antiserum raised against MCR2 that also immunoprecipitates MCR1 (16). As shown in Fig. 3, BRN-1 recognizes the 190-kD MCR1 and the 155-kD MCR2 proteins from a mouse spleen lysate. Only the 190-kD protein is immunoprecipitated by BRN-1 from the cells transfected with the 21 SCR cDNA. The M_r under both reducing and nonreducing conditions is essentially identical to spleen cell–derived MCR1. Pre-immune serum fails to react with either of these two proteins in the spleen or in the transfected cell populations.

Erythrocyte Rosette Formation. Previous experiments have shown that primary spleen cells and cell lines expressing MCR1 are able to form rosettes with Es bearing the mouse C3b fragment and that this reaction is partially inhibited by the mAb 8C12. We used this information to analyze the activities of our rMCR1-expressing K562 cells. As shown in Table 1, these

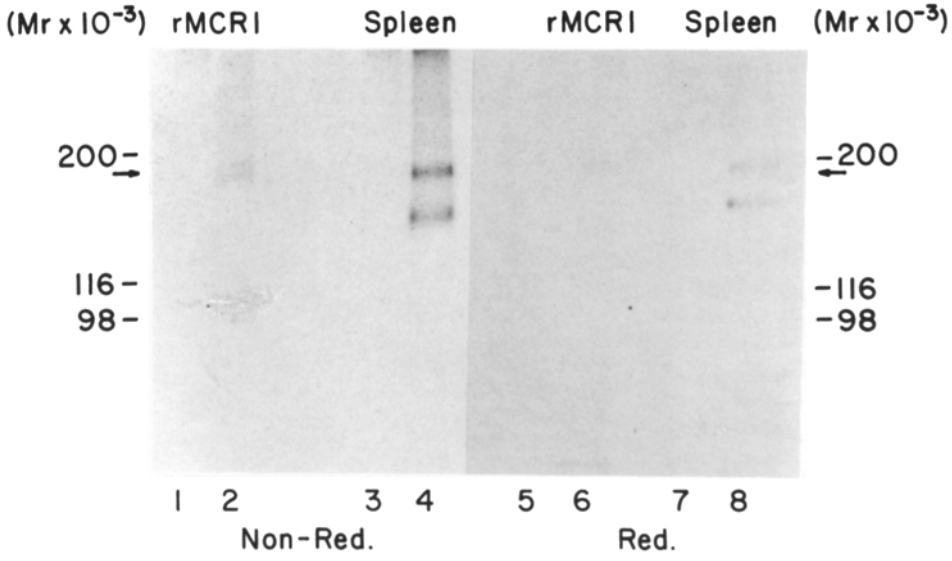

Figure 3. Autoradiograph of 7.5% nonreducing (*left*) and reducing (*right*) SDS-PAGE of surface-labeled and solubilized K562 cells expressing either rMCR1 (lanes *1*, *2*, *5*, and *6*), or BALB/c spleen cells (lanes *3*, *4*, *7*, and *8*), immunoprecipitated with preimmune (lanes *1*, *3*, *5*, and *7*) or immune (lanes *2*, *4*, *6*, and *8*) serum from rabbit BRN-1. (*Arrows*) The specific 190-kD MCR1 band.

Table 1. *E Rosette Formation Mediated by Recombinant Molecules*

Erythrocytes	Cells				
	rMCR1 (sense)	rMCR2 (sense)	rCrry (sense)	rCrry (antisense)	K562
			%		
EAC142	4	1	–*	–	1
EAC1423b	67	32	4	6	3
EAC1423d	15	64	1	2	1
EAC3b + 8C12	42	29	–	–	–
EAC3b + 7G6	18	1	–	–	–
EAC3b + 7E9	65	31	–	–	–
EAC3b + total rat Ig	60	–	–	–	–
EAC3d + 8C12	10	67	–	–	–
EAC3d + 7G6	0	0	–	–	–
EAC3d + 7E9	19	75	–	–	–
EAC3d + total rat Ig	14	–	–	–	–

Percent cells forming rosettes with sheep erythrocytes bearing complement components as noted (human C4b and mouse C3b or C3d), and effect of three mAbs or total rat Ig on rosette formation. Results shown for rMCR1 and rMCR2 were performed at the same time, are representative of many experiments, and were calculated based on ≥ 300 cells counted per condition. Results using rCrry are derived from one experiment based on ≥ 300 cells counted per condition.
* ND.

cells are able to efficiently form rosettes with mouse C3b-bearing Es. These rosettes are partially inhibited by 8C12. In addition, rMCR1-expressing cells also rosette with mouse C3d-bearing Es, although the relative ability is diminished when compared with rMCR2. This reaction is totally inhibited by 7G6, a mAb that completely inhibits C3d-dependent rosette formation with B cell lines and rMCR2-expressing cells (12, 17). 7G6 also partially inhibits rosette formation between rMCR1 and Es bearing mouse C3b. 7E9, another rat mAb that does not possess rosette inhibition activity, and total rat Ig serve as control antibodies. As also shown in Table 1, rMCR2-expressing cells rosette with mouse C3b-bearing Es with a lower efficiency as compared with rMCR1. mAb 7G6, but not 8C12, inhibits the rosette formation between rMCR2 cells and C3b-bearing Es.

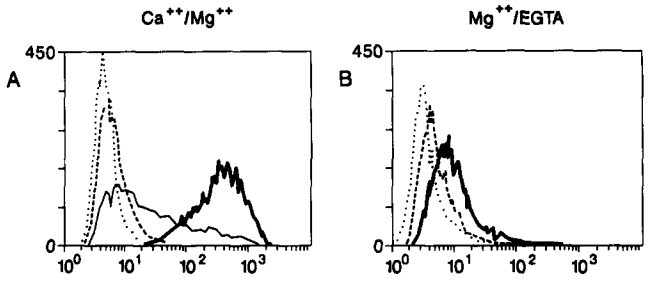

Figure 4. Measurement by flow cytometry of mouse C3 deposition on K562 cells transfected with the rCrry antisense construct. Cells were treated either with rabbit anti-K562 antibody alone (*dotted line*), 20 μl of mouse serum alone (*thin solid line*), anti-K562 antibody plus mouse serum (*thick solid line*), or anti-K562 antibody plus heat inactivated mouse serum (*dashed line*) in either media containing Ca^{2+}/Mg^{2+} (*A*) or media containing Mg^{2+}/EGTA (*B*). *x*-axis is relative fluorescence on a log scale; *y*-axis is cell number.

These results parallel and are entirely consistent with results using spleen cells and cell lines expressing MCR1 and MCR2 (10–12). They suggest that the C3d binding portion of MCR1 is still able to interact with C3d in spite of the extra six SCRs, that the C3d binding site on MCR2 also interacts with mouse C3b, albeit with a lower overall affinity, and that there is an extra C3b binding site in the additional six SCRs found in MCR1. Also of some interest is the finding that rMCR1-expressing cells are unable to efficiently form rosettes with human C4b-bearing Es.

We also analyzed the ability of rCrry-expressing cells to form rosettes with Es bearing these complement components (Table 1). Interestingly, although solubilized p65 has been shown to bind guinea pig C3b-Sepharose, no significant formation of rosettes was detected with mouse C3b-coated Es. This confirms previous observations using cell lines bearing native p65 (19).

Control of Mouse C3 Deposition. We have developed a quantitative assay that measures mouse C3 deposition on the surface of cells as a marker of complement activation. In this assay cells are treated with a specific rabbit polyclonal antibody, forming a complement-activating immune complex, and mouse serum as the source of complement. As Fig. 4 *A* shows, K562 cells expressing the antisense construct treated with an anti-K562 polyclonal antibody in addition to 20 μl

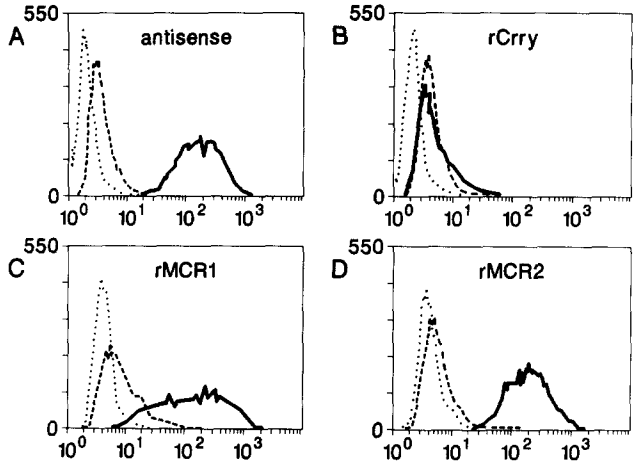

Figure 5. Measurement by flow cytometry of mouse C3 deposition on K562 cells expressing the rCrry antisense construct (*A*), rCrry (*B*), rMCR1 (*C*), or rMCR2 (*D*). Cells were treated with anti-K562 antibody alone (*dotted line*), anti-K562 antibody plus 20 μl of mouse serum (*solid line*), or anti-K562 antibody plus heat-inactivated mouse serum (*dashed line*). *x*-axis is relative fluorescence on a log scale; *y*-axis is cell number.

of mouse serum demonstrate a greatly increased level of C3 on their surface as compared with control cells. Controls include the same cells treated with the anti-K562 antibody in addition to heat-inactivated mouse serum or cells treated with only the anti-K562 antibody. This finding is dependent on the presence of Ca^{2+} in the media as cells incubated with media containing Mg^{2+} and EGTA, or media alone, fail to stain brightly for mouse C3 (Fig. 4 *B*). This indicates that the classical complement pathway is the major determinant of C3 deposition. A slight shift in fluorescence is detected in Mg^{2+}/EGTA medium, which is probably due to alternative pathway activation. The deposition of C3 on K562 cells is dose dependent, begins within the initial 5 min (data not shown), and is still stable and present after 60 min.

As shown in Fig. 4 *A*, mouse serum alone is also capable of increasing C3 deposition on these cells, although to a much smaller amount as compared with our specific antibody and mouse serum combination. This observation is probably due to the presence of low affinity naturally occurring mouse antibody which reacts with the K562 cells and activates complement. Consistent with this, the reaction can be greatly diminished by preabsorbing the sera with K562 cells at 4°C before the first-step incubation with the Ca^{2+}/Mg^{2+} containing media, and is recovered when we add our specific rabbit anti-K562 antibody to the preabsorbed sera (data not shown).

The presence of p65 in a variety of murine tissues has suggested a regulatory function for this protein. To test this possibility, we measured the deposition of mouse C3 on the surface of our rCrry-expressing K562 cells. rCrry-expressing cells which, as previously shown in Fig. 1, have a uniform high level expression of p65, are able to markedly decrease the amount of C3 present on the surface of all cells in the population (Fig. 5 *B*). Cells expressing rMCR1 apparently have little if any ability to control the amount of C3 on their surface as compared with controls (Fig. 5 *C*). Cells expressing rMCR2 (Fig. 5 *D*) are also unable to regulate the amount of C3 deposition. The markedly lower C3 deposition on rCrry-expressing cells is not due simply to a change in kinetics, as analysis of these cells at time points as early as 5 min does not demonstrate C3 deposition at any point (data not shown). The effect is seen over a broad range of amounts of added mouse serum (Fig. 6). As can be seen, the amount of C3 deposition is linear and greatly decreased in rCrry-expressing cells. The effect on complement activation is also not due to altered levels of human intrinsic regulatory proteins, as

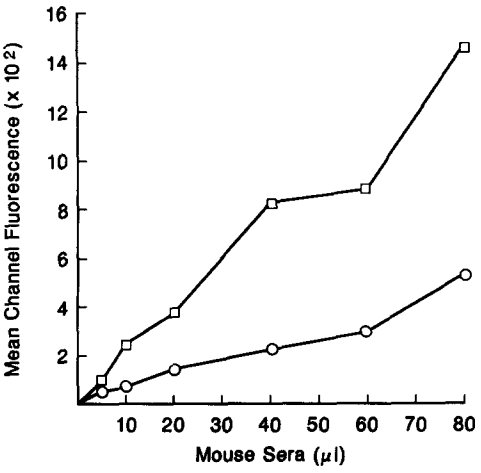

Figure 6. Comparison of input amount of mouse serum (*x*-axis) vs. C3 deposition as determined by mean channel fluorescence (*y*-axis) on rCrry-expressing cells (*open circles*) and antisense construct expressing controls (*open squares*).

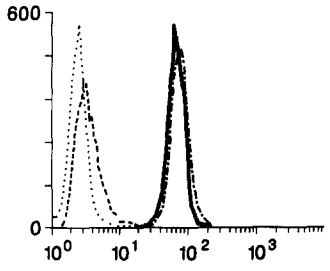

Figure 7. Flow cytometric analysis of MCP expression on transfected K562 cells. rCrry antisense-expressing cells are treated with 7 μg of MOPC (*dotted line*) or 7 μg of anti-MCP mAb (*thick line*). Shown also are rCrry/p65-expressing cells treated with 7 μg of MOPC (*dashed line*) or 7 μg of anti-MCP mAb (*dotted* and *dashed lines*). *x*-axis is relative fluorescence on a log scale; *y*-axis is cell number.

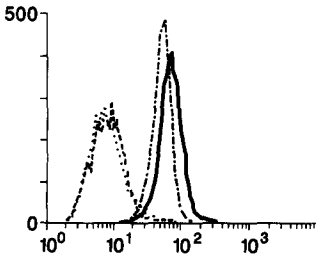

Figure 8. Flow cytometric analysis of DAF expression on transfected K562 cells. rCrry antisense expressing cells are treated with nonimmune rabbit serum (*dotted line*) or immune rabbit anti-DAF serum (*thick line*). Shown also are rCrry/p65-expressing cells treated with nonimmune rabbit serum (*dashed line*) or immune rabbit anti-DAF sera (*dotted* and *dashed lines*). *x*-axis is relative fluorescence on a log scale; *y*-axis is cell number.

the levels of human DAF and MCP are identical in the rCrry-expressing K562 cells as compared with the rCrry antisense construct-expressing K562 cells (Figs. 7 and 8).

Discussion

In these studies we have extended our analysis of the murine counterparts of human complement receptors and regulatory proteins. Our overall purpose has been to relate genes identified as human RCA homologues to characterized mouse proteins which bind C3-Sepharose or mediate rosettes with mouse C3–coated targets. In this report, we present direct proof that the mouse 190-kD MCR1 protein, which previous studies have shown to be the functional homologue of human CR1, is encoded by a transcript generated in the mouse *CR2* (*Cr2*) gene. Our conclusion is based on a number of principles. First, as initially shown by Kurtz et al. (14), the 21 SCR–containing transcript is derived from this gene. Second, our results demonstrate that the protein encoded by this cDNA fulfills functional and immunochemical criteria of the MCR1 protein. This cDNA-derived protein is recognized by the mAb 8C12, which is monospecific for the 190-kD mouse C3-Sepharose binding protein designated MCR1, and has an essentially identical M_r as the splenic MCR1 protein. In addition, it mediates C3b-dependent rosette formation, a reaction that is partially inhibited by mAb 8C12. This is the same functional profile demonstrated by the naturally occurring splenic MCR1 protein. Although our results are consistent with a second C3 binding site within the first six SCRs of this form, expression and analysis of these SCRs alone will be necessary to conclusively demonstrate this point. We have also not yet assayed the cDNA-encoded protein for fluid phase cofactor activity. However, as this is an activity that is directly inhibited in the fluid phase by the 8C12 mAb, we believe the activity will be retained.

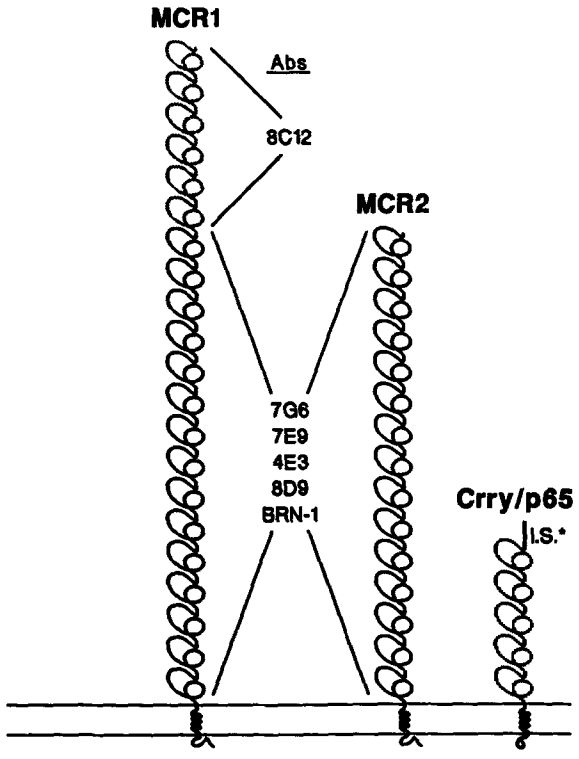

Figure 9. Schematic representation of mouse membrane RCA proteins along with putative sites of antibody reactive. *Intervening sequence present in ~10% of Crry/p65 transcripts.

These results substantiate the principle that both proteins, MCR1 and MCR2, are generated by a single transcriptional unit. In contrast, humans have distinct nonoverlapping genes for human CR1 and CR2, and the proteins are structurally and immunologically distinct. These results also underscore the necessity to develop methods of mouse receptor blockade that are able in vivo to separate activities of each receptor. It has already been noted that no mAbs to MCR2 are unique and that they will also react with and potentially cap or inhibit MCR1. This indicates that the use of soluble receptors or peptides will be necessary to distinguish MCR2 from MCR1 function.

Analysis of ligand specificity by E-rosette studies confirms that MCR1 primarily interacts with mouse C3b and MCR2 with mouse C3d. Of interest, even though MCR1 encodes the mouse C3d binding domain contained within the MCR2 protein, its ability to form rosettes with C3d-coated Es is diminished. This effect of masking the C3d binding site may allow MCR1 to express a biologic role of primarily interacting with C3b and not C3d. This would be an interesting alternative mechanism that achieves the same relative effect as compared with the two separate proteins found in humans.

In these studies we have also analyzed the second human *CR1* genetic homologue, *Crry*. Several lines of evidence have suggested a close relationship between p65 and the *Crry* cDNA gene product. First, p65 was identified by its crossreaction with antiserum raised against human CR1. Second, the *Crry* cDNA is the primary mouse transcript isolated in experiments performed by hybridization of mouse cDNA or genomic libraries with human *CR1* probes. The Crry-predicted protein structure has 60–65% identity with human CR1. Both p65 and Crry have been reported to be present on a wide variety of murine tissues and have been reported to encode membrane bound proteins of 65 and ~70 kD, respectively (19, 21). Although p65 and Crry have demonstrated these characteristics, no previous studies have directly compared the two.

In these studies, we have stably expressed the *Crry* cDNA on a human K562 erythroleukemia cell line background. The sense orientation-transfected cells are specifically and strongly recognized by the anti-p65 antibody. It is very unlikely that this reaction is not specific to p65, or that the antibody is crossreacting with another human RCA protein, because repeated transfection of cells with the Crry antisense construct and selection with neomycin does not result in a cell population that stains with this antibody. Moreover, the anti-p65 antibody does not stain cells expressing rMCR1 or rMCR2. These cells also do not express human CR1, and the levels of human DAF and MCP are equivalent between the sense and antisense construct-expressing cells. Overall, these data constitute the first direct evidence showing that

p65 is encoded by the *Crry* gene. We will now use the designation Crry/p65 to reflect these findings.

To begin to understand the possible functions of Crry/p65 as a mouse RCA protein, we have developed an assay that quantitates the amount of mouse C3 deposited on the surface of K562 cells via the classical pathway. Our data demonstrate that rCrry/p65-expressing K562 cells are capable of intrinsic complement regulatory activity as manifested by a marked decrease in C3 deposition. This inhibition is specific to Crry/p65, as MCR1 and MCR2, which are also mouse C3 binding proteins, have no apparent significant effect on intrinsic C3 deposition.

Interestingly, MCR1 has fluid phase cofactor activity for factor I–mediated cleavage of C3b yet exhibits only minimal or no ability to regulate the amount of C3 deposited on the MCR1-expressing cells. One likely possible explanation for this finding is that this molecule is better suited for extrinsic C3 regulation and is more effective in its interaction with fluid phase complement components or complement fragments bound to immune complexes, as has been suggested for human CR1 (30).

This inhibitory effect of Crry/p65 on intrinsic C3 deposition is due to its ability to interact with the early components of the classical pathway. However, the specific molecular process involved in this regulation is not evident from our studies. Also, we have not yet analyzed its ability to block alternative pathway-dependent C3 deposition. It is likely that Crry/p65 acts to disrupt the C3 convertase. If the activity is similar to that of human DAF or MCP, it will be by the process of decay acceleration or cofactor activity for factor I–mediated C3 and/or C4 cleavage.

Two alternatively spliced *Crry* transcripts have been described, with one of them containing an additional 129 bp of non–SCR-intervening nucleotide sequence between the signal peptide and the first SCR (21). Although identified in all murine cells studied, this form is present in a smaller amount as compared with the most frequent transcript, which we have expressed and which lacks the additional non-SCR sequence. The role of this domain is not known, and further work is needed to elucidate the functional contribution of this region.

In relation to other candidates for mouse intrinsic membrane C3 regulatory proteins, the observation that the nucleotide sequence of Crry/p65 encodes a transmembrane glycoprotein suggests that it is not, at least in this form, the mouse DAF-like protein described by Kameyoshi et al. (18). It is possible that another as yet unidentified transcriptional modification confers upon Crry/p65 a glycolipid anchor; however, the available information suggests that this is likely not the case. Crry/p65 may also belong to a group of ~65-kD C3 binding proteins found on baboon erythrocytes and rabbit macrophages (31–33). However, more information concerning these other molecules is needed before establishing their relationship to Crry/p65.

Overall, it is now clearly apparent that mouse has evolved some remarkably different RCA genes and proteins to perform the apparently necessary biologic roles of complement receptor and intrinsic membrane regulatory protein activities. A summary schematic representation of these three proteins, MCR1, MCR2, and Crry/p65, along with their sites of antibody reactivity are shown in Fig. 9. The genetic relationships between these genes and their human homologues have been extensively discussed (13–17, 20–23). The two primary mechanisms proposed to account for the differences in gene structures between species are continued evolution facilitated by unequal crossing over of repeat regions and deletions within the mouse RCA locus. Both mechanisms have likely played significant roles in this process. Analysis of the RCA locus of other species should prove very informative in this regard.

Finally, both functional blockade and analysis of the expression of MCR1, MCR2, and Crry/p65 in mouse models of inflammation should allow an increased understanding of the role of these proteins and their receptor or intrinsic membrane complement regulatory activities in these processes. Analysis of mice with specific deletions of these genes will allow a more complete understanding of their roles in development of the immune system and in specific immune responses.

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