

# Mouse Complement Regulatory Protein Crry/p65 Uses the Specific Mechanisms of Both Human Decay-accelerating Factor and Membrane Cofactor Protein

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## Summary

Normal host cells are protected from the destructive action of complement by cell surface complement regulatory proteins. In humans, decay-accelerating factor (DAF) and membrane cofactor protein (MCP) play such a biologic role by inhibiting C3 and C5 convertases. DAF and MCP accomplish this task by specific mechanisms designated decay-accelerating activity and factor I cofactor activity, respectively. In other species, including mice, structural and/or functional homologues of these proteins are not yet well characterized. Previous studies have shown that the mouse protein Crry/p65 has certain characteristics of self-protecting complement regulatory proteins. For example, Crry/p65 is expressed on a wide variety of murine cells, and when expressed on human K562 erythroleukemic cells, it prevents deposition of mouse C3 fragments on the cell surface during activation of either the classical or alternative complement pathway. We have now studied factor I cofactor and decay-accelerating activities of Crry/p65. Recombinant Crry/p65 demonstrates cofactor activity for factor I-mediated cleavage of both mouse C3b and C4b. Surprisingly, Crry/p65 also exhibits decay-accelerating activity for the classical pathway C3 convertase strongly and for the alternative pathway C3 convertase weakly. Therefore, mouse Crry/p65 uses the specific mechanisms of both human MCP and DAF. Although Crry/p65, like MCP and DAF, contains tandem short consensus repeats (SCR) characteristic of C3/C4 binding proteins, Crry/p65 is not considered to be a genetic homologue of either MCP or DAF. Thus, Crry/p65 is an example of evolutionary conservation of two specific activities in a single unique protein in one species that are dispersed to individual proteins in another. We propose that the repeating SCR motif in this family has allowed this unusual process of evolution to occur, perhaps driven by the use of MCP and DAF as receptors by human pathogens such as the measles virus.

Normal cells are protected from the inadvertent destructive action of complement primarily by the activities of intrinsic membrane regulatory proteins (1). These regulatory proteins act either at the early C3/C5 convertase steps or during the later assembly of the membrane attack complex (MAC)<sup>1</sup>. In humans, two membrane regulatory pro-

teins are present that act on C3/C5 convertases. These proteins are decay-accelerating factor (DAF, CD55) (2) and membrane cofactor protein (MCP, CD46) (3).

DAF and MCP each use a unique mechanism to accomplish self protection of cells. DAF acts by blocking convertase assembly and accelerating the spontaneous decay of C2a and Bb from C4b and C3b, respectively (4). MCP, conversely, has no decay-accelerating activity but rather acts as a required cofactor for factor I-mediated cleavage of C3b and C4b (5). Factor I-cleaved forms of C3b and C4b have no further hemolytic or complement activating capacity. Using these two distinct mechanisms, DAF and MCP together exert a substantial capacity to block further complement activation on cell

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<sup>1</sup> Abbreviations used in this paper: CR, complement receptor; DAF, decay-accelerating factor; GPI, glycosphosphatidylinositol; LHR, long homologous repeat; M, mouse; MAC, membrane attack complex; MCP, membrane cofactor protein; PNH, paroxysmal nocturnal hemoglobinuria; RCA, regulators of complement activation; SCR, short consensus repeat.

membranes by either the classical or alternative complement pathway.

The biologic importance of membrane complement inhibitory proteins has been demonstrated by observations in a number of human diseases. One example is paroxysmal nocturnal hemoglobinuria (PNH) (6). PNH is an acquired somatic cell disorder manifest in bone marrow lineage cells. Somatic mutations in the *PIG-A* gene found on the X chromosome result in absent or substantially lower levels of glycosylphosphatidylinositol (GPI)-anchored proteins in patients with PNH (7). Two membrane complement regulatory proteins, DAF and CD59 (a MAC inhibitor) use GPI anchors for membrane attachment (8, 9). The absence of DAF and CD59 in PNH results in the spontaneous hemolysis of erythrocytes due to the relatively unchecked activity of the alternative complement pathway.

A more complete understanding of the biologic roles of these proteins would result from the analysis of their homologues in other species, especially the mouse. DAF and MCP are members of a gene family designated the regulators of complement activation (RCA) (10). RCA genes are characterized by the presence in the derived protein sequence of a repetitive motif ~60–70 amino acids in length that is designated a short consensus repeat (SCR). SCRs contain four invariant cysteines and one tryptophan in addition to other highly conserved but not invariant amino acids. In the mouse, analysis of the RCA genes and their products has revealed a number of significant differences as compared with humans. In particular, no genes or cDNAs with enough nucleotide similarity to either DAF or MCP have been able to be identified in cross species screening despite many attempts (11, 12). In addition, two other RCA proteins, complement receptors 1 and 2 (CR1 and CR2), are derived by different molecular mechanisms (12–14). Human CR2 and CR1 are the products of unique genes (10), while mouse CR2 (MCR2) and a CR1 homologue (MCR1) found on B lymphocytes and follicular dendritic cells (FDCs) are the products of alternatively spliced RNA derived from the single *Cr2* gene.

With regard to potential DAF and MCP homologues, based on a number of lines of evidence we have previously proposed that one mouse RCA family gene, *Crry*, encodes a functional homologue of mouse DAF or MCP (11, 12, 15). The *Crry* gene was originally identified using cDNA probes for human CR1 (16, 17). As opposed to human CR1, though, *Crry*-derived mRNA and protein are widely distributed (15, 16, 18). Expression of the recombinant *Crry* cDNA in the human K562 cell line results in a protein recognized by a rabbit polyclonal antibody prepared using human CR1 as an immunogen (12). This antisera had been used previously to identify and partially characterize a mouse protein called p65 (19). Because of these results, we have since designated the protein product of the *Crry* gene *Crry/p65*. We have also found that *Crry/p65* cannot act as a receptor for mouse C3b- or human C4b-coated erythrocytes (12). However, recombinant *Crry/p65* protein is able to block activation of both the mouse classical (12) and alternative (20) complement pathways. Several rat mAbs that react with *Crry/p65* can re-

verse classical complement pathway control on cell membranes in which recombinant *Crry/p65* is expressed (15).

The specific mechanisms by which *Crry/p65* can control complement activation have not been previously defined. Herein, we demonstrate that *Crry/p65* has both decay-accelerating and cofactor activities. Thus, this one mouse protein encompasses the complement regulatory activities of both human DAF and MCP. Based on these results and a further comparative analysis of mouse and human RCA gene structures, we propose that the repetitive nature of this SCR-containing family has allowed this unusual evolutionary process to occur.

## Materials and Methods

**Buffers and Reagents.** The following buffers were used: isotonic veronal buffer, pH 7.4, containing 72.7 mM NaCl, 2.5% dextrose, 0.1% gelatin, 0.15 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub> (DGVB); and 0.1% gelatin-veronal-buffered saline, pH 7.4, containing 10 mM EDTA (EDTA-GVB). Guinea pig C1 and C2 (21), human C3 and C4 (22), mouse factors H (23) and I (24), and mouse C3 (23) were purified as described elsewhere. Mouse C4 was partially purified from plasma by polyethylene glycol (PEG) fractionation, QAE-Sephadex A-50 and Mono Q column chromatography. C4 was monitored by hemolytic assay, SDS-PAGE and Ouchterlony analysis with anti-mouse C4 antiserum. Purity of the final preparation can be evaluated from Fig. 2. Mouse factor B was also partially purified from the same plasma. In fractions obtained from the above mentioned QAE-Sephadex A-50 column, factor B was detected by hemolytic assay. Factor B was further purified by DEAE-Sephacel column. Partially purified factor B was cleavable by factor D plus C3b, and its identity was confirmed by amino acid sequence.

**Purification of Recombinant Soluble *Crry/p65* (rs*Crry/p65*).** rs*Crry/p65* was purified to homogeneity by sequential column chromatography as previously described (20) from the supernatant of *Trichoplasia ni* cells (Invitrogen Corp., San Diego, CA) infected with baculovirus encoding a soluble form of *Crry/p65*. rs*Crry/p65* includes the complete extracytoplasmic domain 5 SCRs of *Crry/p65* and is truncated after the last cysteine of SCR 5. Before use, rs*Crry/p65* was dialyzed into DGVB buffer.

**Extracts of K562 Erythroleukemic Cells Expressing *Crry/p65*, MCR1 or MCR2.** K562 cells transfected with *Crry* cDNA, antisense *Crry* cDNA, MCR1 cDNA, and MCR2 cDNA as described previously were used as sources of recombinant proteins (12). Transfectants were cultured in RPMI 1640 containing 10% FCS and 400 µg/ml G418 (Geneticin; GIBCO BRL, Gaithersburg, MD). For extraction the cells were washed with PBS and incubated at 10<sup>7</sup>/ml in PBS containing 0.5% NP-40 and 1 mM PMSF at room temperature for 15 min followed by centrifugation at 1,500 rpm for 10 min and then 12,000 rpm for 5 min. The supernatants were stored frozen at -80°C.

**Assay of Factor I Cofactor Activity.** C3 and C4 were labeled with <sup>125</sup>I (DuPont, Wilmington, DE) in IODOGEN (Pierce, Rockford, IL)-coated test tubes (25). The preparations had specific activities of 10<sup>6</sup>–5 × 10<sup>6</sup> cpm/µg protein and were active after iodination. Antibody-sensitized sheep erythrocytes (EA cells) bearing C1 (EAC1 cells) were prepared by incubation of EA cells (10<sup>8</sup>/ml) with guinea pig C1 (200 U/ml) for 15 min at 30°C. EAC14 cells bearing human or mouse C4b were prepared by incubating EAC1 cells (10<sup>8</sup>/ml) with an equal volume of human C4 or mouse C4 (10 µg/ml) for 20 min at 30°C.

<sup>125</sup>I-labeled fluid phase mouse C3b was obtained in the supernatant by incubating <sup>125</sup>I-C3 at 0.5 μg/ml with 5 × 10<sup>7</sup> EAC142 cells/ml in DGVB at 30°C for 15 min. A mixture of <sup>125</sup>I-labeled C3b (100 ng/ml), factor I (200 ng/ml), and cell extracts (4 × 10<sup>5</sup> cell equivalents/ml) was incubated for 50 min at 37°C. Cleavage of the α' chain of C3b was assessed by SDS-PAGE under reducing conditions followed by autoradiography.

Partially purified mouse C4 was radiolabeled and incubated with EAC1 cells to convert C4 to C4b. The resulting EAC14 cells were hypotonically lysed and the ghosts were extracted with 0.5% NP-40. This extract was used as a source of cell-bound C4b. The supernatant obtained after incubation of C4 with EAC1 cells was used as a source of fluid-phase C4b. These radiolabeled C4bs were incubated as above with mouse factor I in the presence of K562 detergent extracts as a source of cofactor. To eliminate any activity of K562-derived MCP, anti-MCP in mAb M177, previously shown to block human MCP cofactor activity in vitro (26), was included in the cofactor assays at 10 μg/ml.

**Assay of Decay-accelerating Activity for the Classical Pathway C3 Convertase.** EAC142 cells were prepared by incubating EAC14 cells bearing mouse C4b with guinea pig C2 for 5 min at 30°C. The EAC142 cells were incubated, for various durations of time as shown in Results, at 30°C in the presence or absence of rsCrry/p65 to allow decay of C4b2a. The residual C4b2a sites were assessed by incubating the cells with guinea pig serum in EDTA-DGVB as a source of C3-C9 for 60 min at 37°C.

**Assay of Decay-accelerating Activity for the Alternative Pathway C3 Convertase.** EAC3b cells were prepared by incubating EAC142 cells (5 × 10<sup>8</sup>/ml) bearing human C4b and guinea pig C2a with mouse C3 (70 μg/ml) for 30 min at 30°C followed by incubation with EDTA-containing buffer at 37°C for 2.5 h to inactivate C1 and C2a. EAC3b cells (5 × 10<sup>8</sup>/ml) were incubated in MgEGTA-GVB with mouse B (30 μg/ml) and human D (2 μg/ml) for 5 min at 30°C to prepare EAC3bBb cells.

EAC3bBb cells were incubated for various times at 30°C in the presence or absence of rsCrry/p65. The residual C3bBb sites were assessed by incubating the cells with rat serum in EDTA-DGVB as a source of C3-C9 for 60 min at 37°C.

**Mapping of Mouse Cr2 Exons Encoding SCRs 1-6 of MCR1.** A genomic clone (λ13) was isolated from a λ fix II library of murine genomic DNA (14) with an MCR1 SCR 1-6 containing cDNA probe. λ13 DNA was digested with several restriction enzymes and subjected to Southern blot analysis using MCR1 SCR-specific 5' <sup>32</sup>P-labeled oligonucleotides (Table 1). λ13 DNA fragments were subcloned with the pBSKS vector (Stratagene, San Diego, CA) and their respective coding regions were sequenced using a strategy previously used to map exon-intron junctions of human CR2 (27) and CR1 (28). Briefly, oligonucleotides from the 5' and 3' ends of each SCR were used to sequence across the 3' and 5' junctions, respectively. Split exons were confirmed, where necessary, using oligonucleotides prepared using intron sequence. Exons encoding SCR 1-5 and the NH<sub>2</sub>-terminal portion of SCR 6 were characterized.

## Results

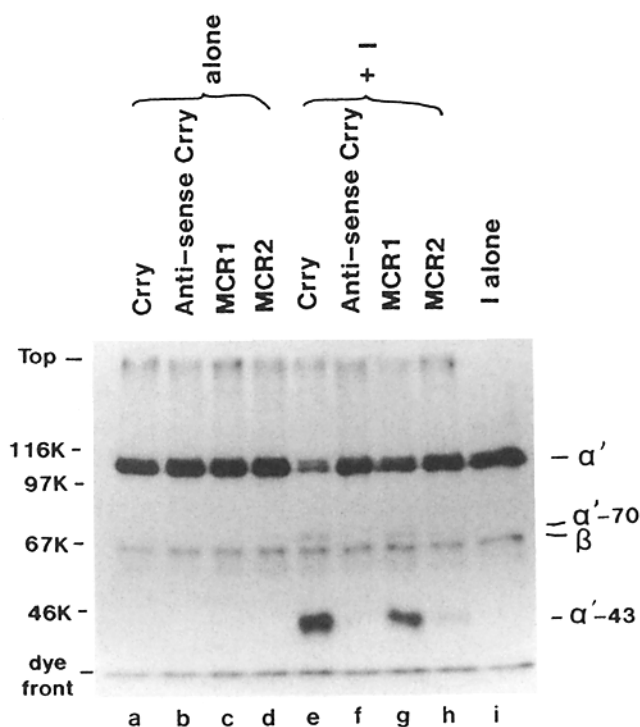
**Factor I Cofactor Activity of Crry/p65.** We initially expressed recombinant Crry/p65 in K562 human erythroleukemic cells and used their detergent extract as a source of Crry/p65. As a negative control, we used detergent extracts of K562 cells expressing Crry cDNA in the antisense orientation. For comparison, we also used detergent extracts of K562 cells expressing MCR1 and MCR2 cDNAs as sources of MCR1 and MCR2, respectively. To further assure the origin of the cofactor activity, human MCP blocking mAb M177 was included in the reactions.

As shown in Fig. 1, mouse factor I cleaved radiolabeled

**Table 1.** Cr2-derived Oligonucleotides for Exon-Intron Junctional Analysis

Oligonucleotide	Sequence	Exon
MC2.49	TGTAAGTTGCTGCCAAAGTA	1
MC2.56	ACAGAACTGCTGAGCATCTGA	1
MC2.50	CATGTATGAATCCTCAAGAA	2A
MC2.101	ATTACAAGAATACGTAATTG	2A
MC2.102	CGACTCATTGGTGACTCGTC	2B
MC2.57	ACAAAGAGGCATATCATTAT	2B
MC2.51	GAGTCACCTCCAGCCATCTC	3,4
MC2.58	TACACTGAGGAGGTGGACTAT	3,4
MC2.63	GCAGAGACAGCTTTTTCTATGGGATGGTAGTAACTT	3,4
MC2.52	ATGCCAGAATTGAAAATGG	3,4
MC2.65	CTAGAATAGCATGGTGCCAGCCAAACAGCAAATGGA	3,4
MC2.53	TGTCTACCACCTCAAAATAT	5
MC2.60	ACATGTCGGGACTGTATTGCT	5
MC2.54	TGTGATGCAATTCCAAACCA	6A
MC2.103	CACAAACAAAGGAAACCTCT	6A

Oligonucleotides derived from the MCR1 cDNA nucleotide sequence used to identify genomic subclones and sequence across exon-intron junctions shown in Table 2. These sequence data are taken from reference 13.

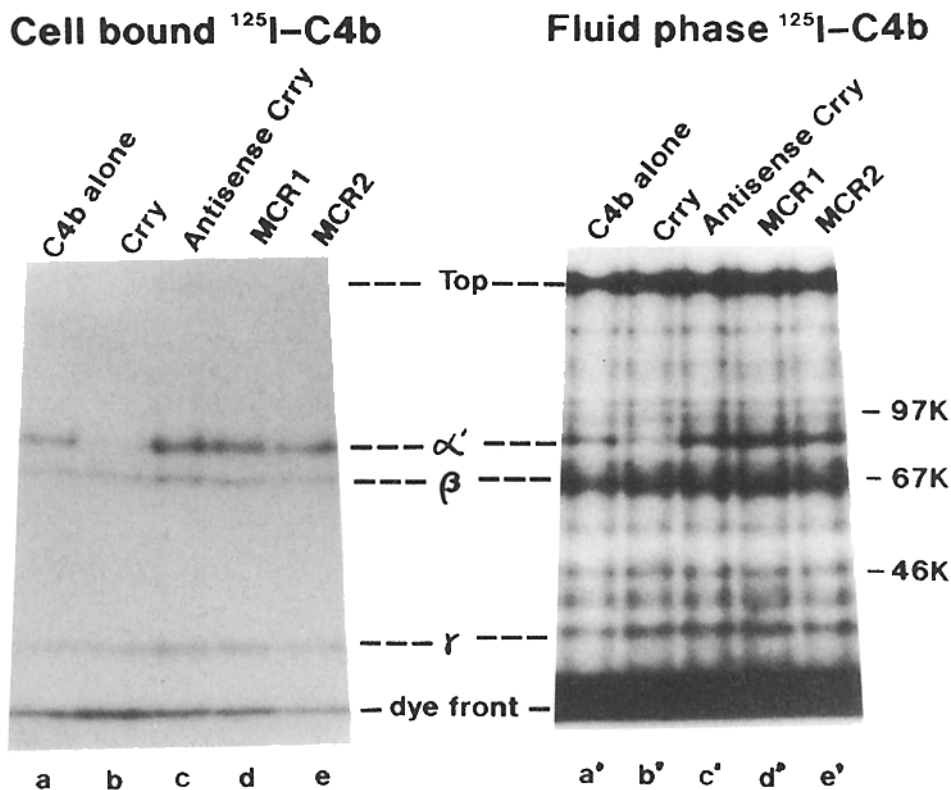


**Figure 1.** Factor I cofactor activity of Crry/p65 in the cleavage of C3b. Radiolabeled mouse C3b was incubated with one of the cofactors alone (lanes *a-d*), or with a cofactor source plus mouse factor I (lanes *e-h*), or with factor I alone (lane *i*). Cleavage of the  $\alpha'$  chain of C3b was assessed by SDS-PAGE under reducing conditions with a 7.5% gel followed by autoradiography. The cofactor sources are extracts of cells expressing Crry/p65 (lanes *a* and *e*), antisense construct of Crry (lanes *b* and *f*), MCR1 (lanes *c* and *g*), or MCR2 (lanes *d* and *h*). Molecular sizes are on the left.

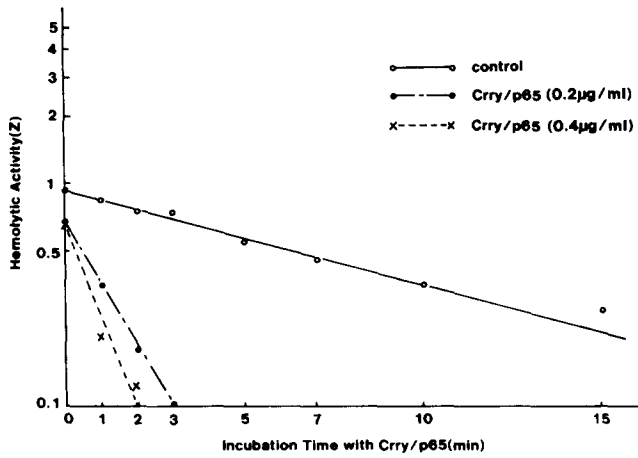
mouse C3b in the presence but not in the absence of Crry/p65 (Fig. 1, lanes *e* and *i*, respectively). This cofactor activity must be specific to Crry/p65 because the extract of cells expressing the anti-sense construct of Crry has no activity (Fig. 1, lane *f*). MCR1 showed strong and MCR2 showed weak cofactor activities (Fig. 1, lanes *g* and *h*), consistent with previous reports using purified native proteins (29-31). The cleavage products of the  $\alpha'$  chain of mouse C3b are the same with Crry/p65 and MCR1, i.e.,  $\alpha'$  70 and  $\alpha'$  43.

We next tested factor I cofactor activity of Crry/p65 in the cleavage of C4b. As shown in Fig. 2 (*left*), the  $\alpha'$  chain of cell-bound C4b was cleaved by factor I in the presence of the extract of the cells expressing Crry/p65 (Fig. 2, lane *b*) but not the extract of the cells expressing antisense Crry (Fig. 2, lane *c*). The cleavage products of the  $\alpha'$  chain apparently migrated with the dye-front. MCR1 and MCR2 had no detectable cofactor activity in C4b-cleavage (Fig. 2, lanes *d* and *e*). Analysis with fluid phase C4b gave a similar result (Fig. 2, *right*) and demonstrated that the  $\alpha'$  chain of C4b was cleaved only with Crry/p65 and factor I (Fig. 2, lane *b'*).

**Decay-accelerating Activity of Crry/p65.** To test decay-accelerating activity of Crry/p65, it was necessary to use a strategy that would exclude hemolysis due to the presence of detergent during the study. To accomplish this, we used purified recombinant soluble Crry/p65 (rsCrry/p65) that lacks transmembrane and intracytoplasmic domains and is, therefore, soluble in detergent-free conditions. Sheep erythrocytes bearing the classical pathway C3 convertase that contains mouse C4b (EAC14mo2 cells) were then incubated with buffer in the presence or absence of rsCrry/p65. At timed intervals an aliquot was taken and further incubated with, as a source of



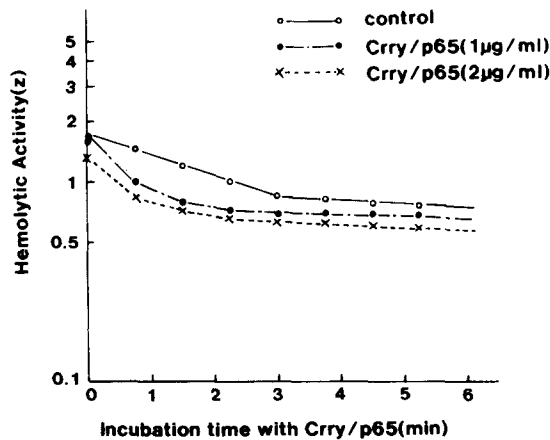
**Figure 2.** Factor I cofactor activity of Crry/p65 in the cleavage of C4b. Radiolabeled cell-bound (*left*) and fluid-phase (*right*) mouse C4b were incubated with mouse factor I and a cofactor. Cleavage of the  $\alpha'$  chain of C4b was assessed by SDS-PAGE under reducing conditions with a 10% gel followed by autoradiography. (Lanes *a-e*) Cell-bound C4b; (lanes *a'-e'*) fluid-phase C4b; (lanes *a* and *a'*) C4b alone; (lanes *b* and *b'*), Crry/p65 plus factor I; (lanes *c* and *c'*) extract of cells expressing antisense construct of Crry plus factor I; (lanes *d* and *d'*) MCR1 plus factor I; (lanes *e* and *e'*) MCR2 plus factor I. Molecular sizes are on the right.



**Figure 3.** Acceleration of decay of the classical pathway C3 convertase by recombinant soluble Crry/p65. Sheep red cells bearing C4b2a complexes (EAC142 cells) were incubated with DGVB (O), buffer containing 0.2  $\mu\text{g/ml}$  Crry/p65 (●) or buffer containing 0.4  $\mu\text{g/ml}$  Crry/p65 (X) at 30°C for 0–15 min to allow decay of the C3 convertases. After the decay, remaining C3 convertase was assessed by incubating the cells with C3-C9 for 1 h at 37°C followed by centrifugation and spectrophotometric measurement of released hemoglobin.

C3-C9, guinea pig serum diluted in EDTA-containing buffer. This allowed us to evaluate by hemolysis the C3 convertase activity remaining on the erythrocyte surface at the time of sampling. As shown in Fig. 3, rsCrry/p65 accelerated decay of the classical pathway C3 convertase strongly, decreasing the half-life from 7 min to 1.2 min at 0.2  $\mu\text{g/ml}$  and to 0.7 min at 0.4  $\mu\text{g/ml}$ .

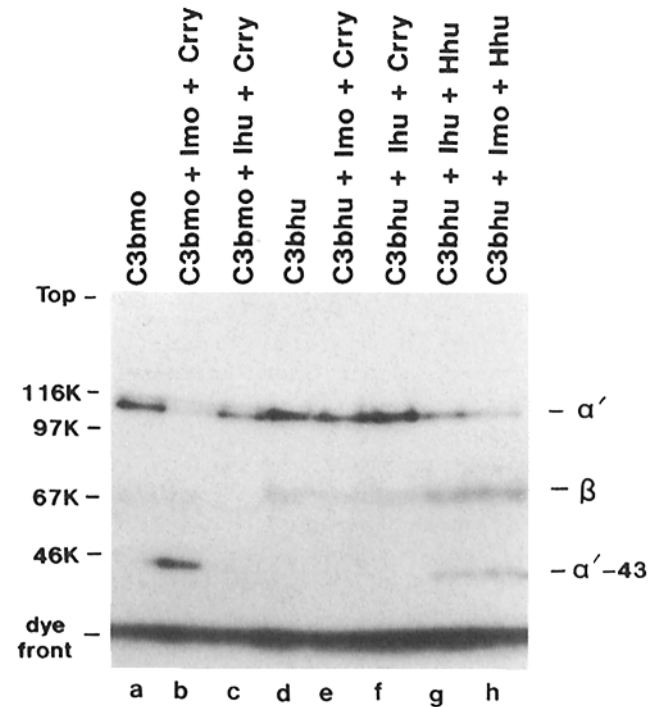
We next tested decay-accelerating activity of Crry/p65 on the alternative pathway C3 convertases. Sheep erythrocytes bearing C3bBb complexes that consist of mouse C3b and mouse Bb (Ec3bBb cells) were incubated with buffer in the



**Figure 4.** Acceleration of decay of the alternative pathway C3 convertase by Crry/p65. Sheep red cells bearing C3bBb complexes (EC3bBb cells) were incubated with Mg-EGTA-GVB (O) or buffer containing 1  $\mu\text{g/ml}$  Crry/p65 (●) or 2  $\mu\text{g/ml}$  Crry/p65 (X) at 30°C for 0–10 min to allow decay of the C3 convertases. After the decay, remaining C3 convertase was assessed by incubating the cells with C3-C9 for 1 h at 37°C followed by centrifugation and spectrophotometric measurement of released hemoglobin.

presence or absence of rsCrry/p65. At timed intervals an aliquot was taken into a source of C3-C9, rat serum diluted in EDTA-containing buffer, to evaluate the residual convertase activity by hemolysis. Without Crry/p65, the alternative C3 convertase decayed with a half-life of 3 min initially. After 3 min, stabilization of the convertase occurred most likely by properdin present in the samples of complement components used to prepare EC3bBb cells. With rsCrry/p65, the decay curves were also not straight. Nevertheless, it is apparent that the decay was accelerated by rsCrry/p65 (Fig. 4). However, ten times as much rsCrry/p65 was necessary for significant acceleration of decay of the alternative pathway C3 convertase than for the classical pathway C3 convertase.

**Species Specificity of Crry/p65 Activity.** Human complement regulatory proteins that protect host cells often show species specificity, that is they are inactive against heterologous complement. We tested whether Crry/p65 has this characteristic of self-protecting complement regulatory proteins. As shown in Fig. 5, the combination of mouse factor I and Crry/p65 did not cleave human C3b (Fig. 5, lane e). Since mouse factor I had ability to cleave human C3b with human factor H (Fig. 5, lane h), this indicates that Crry/p65 is incompatible with human C3b. The combination of human factor I and Crry/p65 did not cleave either mouse C3b or



**Figure 5.** Species specificity of Crry/p65. Species specificity of cofactor activity of Crry/p65 was tested using combinations of C3b and factor I of human (hu) and mouse (mo) origins. Radiolabeled mouse C3b (lanes a–c) and radiolabeled human C3b (lanes d–h) were incubated with Crry/p65 and either mouse or human factor I at 37°C for 1 h. Cleavage of the  $\alpha$  chains was then assessed by SDS-PAGE and autoradiography. (Lanes a and d) C3b alone; (lanes b and e) mouse factor I and Crry/p65; (lanes c and f) human factor I and Crry/p65; (lane g) human factor I and human factor H; (lane h) mouse factor I and human factor H.

human C3b (Fig. 5, lanes *c* and *f*), indicating that Crry/p65 is incompatible with human factor I. Thus, cofactor activity of Crry/p65 is restricted to homologous complement.

## Discussion

In these studies we have characterized the molecular mechanisms of intrinsic membrane complement regulation by the Crry/p65 protein of the mouse RCA family. Surprisingly, this one mouse protein, Crry/p65, uses the specific complement regulatory mechanisms of both human DAF and MCP. Crry/p65 acts as a decay-accelerator in addition to a factor I cofactor, and it can perform these activities in both C3 and C4 dependent systems. The net result of these regulatory activities is self-protection of cells from untoward activation of either the classical or alternative pathway. Crry/p65 is a widely distributed protein, as are DAF and MCP in toto. Crry/p65 also demonstrates species restriction. Based on these similarities in function and sites of expression, in addition to the lack of detection of DAF and MCP related genes, we believe that Crry/p65 performs many if not all of the same biologic roles in mouse as DAF and MCP in humans. In this light, the lack of detectable crosshybridization in mouse by human DAF and MCP cDNAs is not entirely surprising.

We do not currently have an explanation for one previous study in which a purified GPI-anchored mouse erythrocyte protein was reported to have decay-accelerating activity for both mouse C3/C5 convertases and the human classical pathway C3 convertase (32). A polyclonal antibody reported to be reactive with that protein failed to stain our K562 cells expressing Crry/p65 (Okada, H., and V. M. Holers, unpublished observations). In addition, our previous studies have not detected release of Crry/p65 from mouse erythrocytes or splenocytes by PIPLC under the same conditions used to release Thy-1 in parallel (15). Resolution of this issue awaits the identification of the gene encoding this putative GPI-anchored complement regulatory protein followed by a comparison to Crry/p65. Pertinent to this issue, our studies cannot rule out the presence of other membrane proteins in mouse with complement regulatory activity acting at the C3/C5 convertase steps.

In these studies, we have also compared the activities of Crry/p65 with MCR1. There are important differences between the two proteins. Crry/p65 demonstrates cofactor activity for mouse factor I-mediated cleavage of both mouse C3b and C4b. In addition, Crry/p65 protects cells from C3 deposition resulting from both classical and alternative pathway activation. In our current study, however, MCR1 manifests no detectable cofactor activity for mouse factor I-mediated cleavage of mouse C4b, in spite of demonstrating this activity for mouse C3b under similar conditions. Membrane-bound MCR1 also does not effectively protect cells from classical pathway activation (12). These observations provide further support for our hypothesis that Crry/p65 is the primary intrinsic membrane complement regulatory protein in mouse.

We cannot yet in these studies determine a specific activity for Crry/p65 relative to MCR1. However, the absence of C4b cleavage by MCR1 when C3b cleavage is manifest by both

MCR1 and Crry/p65, and the easily demonstrable C4b cleavage by Crry/p65, is an important consideration in comparing the relative C3 versus C4 regulation by these two proteins. These results suggest that Crry/p65 is overall a more potent complement regulatory protein. The lack of efficient factor I cofactor activity for mouse C4b exhibited by MCR1 is also interesting in light of its apparent ability to act as a receptor for rat C4b-coated erythrocytes (33).

With these experimental findings, obvious questions include both how and why this difference between the mouse and human RCA gene families could have occurred during evolution. We believe that a comparison of the structures of the human and mouse genes provides a rational mechanism by which two evolutionary paths could have been taken. The structural relationships of RCA proteins and possible evolutionary relationships of human and mouse RCA genes encoding CR1, CR2, and Crry/p65 have been discussed in previous publications (13, 14, 18). These analyses have demonstrated a relatively high degree of protein and nucleotide sequence conservation and have suggested a linked evolution. For instance, the first four SCRs of the Crry/p65 protein are most similar in sequence to the first four SCRs of both MCR1 and human CR1. The 5th SCR of Crry/p65 is very similar to the 30th SCR of human CR1, both of which are the most membrane proximal SCRs. Human CR2 and CR1 have sequence similarity (34, 35). In addition, human CR2 and MCR2 sequences are ~70% identical (13, 14), and the human CR2 gene contains nonexpressed nucleotide sequences apparently derived from expressed exons within the *Cr2* gene encoding the first six SCRs of MCR1 (36). The mouse RCA genes encoding *Cr2* and *Crry* have also apparently undergone a translocation and/or inversion resulting in the movement of these two genes ~40 cM away from the comparable location in the human genome (37).

For the present comparison, we have taken further advantage of one characteristic feature of SCR-containing genes that has greatly facilitated the analysis of the evolution of RCA genes. That feature is the different type of exon that encodes each SCR. SCRs are found to be encoded either on a single exon, as a pair of SCRs on a single exon, or as a split SCR encoded on two separate exons (10, 27). In each exon type in all of the RCA genes, the phase of exon-intron junctions are completely conserved. To obtain this information with the goal of further understanding the evolution of the human and mouse RCA genes, we have determined the rest of the exon structure of the *Cr2* gene encoding the first six SCRs of MCR1.

Results of this analysis are included in Table 2 and in schematic fashion in Fig. 6. The *Cr2* SCR organization is shown compared with the previously determined *Crry* gene organization (38) in addition to human CR1 (39), CR2 (27), DAF (40), and MCP (3). In this figure, we have included 22 SCRs in the mouse *Cr2* gene in order to include the alternatively spliced exon in the human CR2 gene, as it is likely that at least a remnant of that exon is present in the mouse (14). What is readily apparent is the presence of a module encoding four SCRs (Fig. 6, *bracket*). This module consists of a motif of exons encoding a single SCR followed by a

**Table 2.** *Cr2* Exon-Intron Junction Sequences

Exon	Junctions
1	tatttctag/GT CAG ... AAA C/gtgagtacttct
2A	cccttgtag/GT AAA ... CAA GG/gtgagtaggcag
2B	tagttctag/A TAT ... GAA T/gtagataaaatg
3,4	cctccaaag/CT ATT ... ATG G/gtaagttgggcc
5	ttcttgtag/GA TGT ... GAA G/gtgcttagagct
6A	tttcctag/TG AAA ... TTA GG/gtgagtgtgaag

Exon-intron junctions of exons encoding the first 5.5 SCRs of MCR1. These sequence data are available from EMBL/GenBank/DBJ under accession numbers U171123-8.

split SCR and then two fused SCRs and is found in each gene except DAF and MCP. Not surprisingly, the Crry/p65 module is in the first four SCRs and includes the region of most sequence similarity to human CR1 and MCR1. This module is present as the first 4 SCRs of human CR1 long homologous repeat (LHR) A, in which the C4b-binding site of human CR1 is found (41, 42). C3b-binding sites are found in comparable sites of the highly repetitive LHRs B and C, and this module is also found at the same two sites. The first four SCRs of MCR1 also consist of this module and contain the C3b/C4b binding site in addition to the C3b cofactor site (33, 43).

We propose that this four SCR-containing module is a primordial C3b/C4b binding element that apparently evolved before speciation separating humans and mice. Subsequently, this module has evolved into either a C3b and/or C4b receptor, or a C3/C4 regulatory protein, depending on the species and

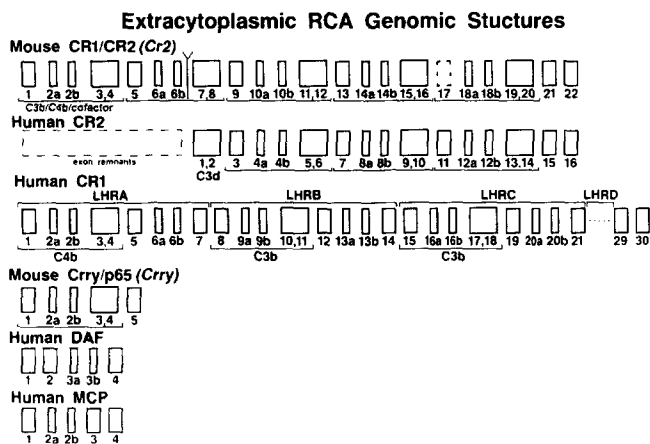
context in which it is found. In some locations, such as LHR D of human CR1 and the membrane proximal modules of MCR1 and CR2, no C3b or C4b binding is detected. However, one module of CR2 demonstrates C3d binding within one fused SCR exon (Fig. 6). Crry/p65 demonstrates the most extensive binding and regulatory activity found to date that is likely to be conserved within a single modular element.

Only one other member of the RCA family, human CR1, has both decay-accelerating and factor I cofactor activity. Although the C3 and C4 binding and regulatory sites are non-overlapping and contained in separate modules, it is of some interest also that Crry/p65 was first recognized by its cross-reactivity with anti-human CR1 antibody and its crosshybridization with human CR1 cDNA.

Thus, our results strongly support the hypothesis that in mouse and in humans two different evolutionary paths were taken. The apparent loss of MCP and DAF in the murine lineage may have, in part, been driven by the opportunistic use of MCP and/or DAF by human pathogens. In humans, MCP is the primary receptor for the measles virus (44, 45), and DAF may be an *Escherichia coli* receptor (46). Certain mouse cells can only be infected by the measles virus after transfection of recombinant MCP (45). Therefore, driven by this type of evolutionary pressure, a partial duplication of *Cr2* may alone have eventually generated the Crry/p65 molecule that could provide the same self-protection on cell membranes of mice as DAF and MCP do in humans.

We cannot yet devise a molecular model of evolution that incorporates all of the RCA family membrane bound receptors and regulatory proteins. Molecular analyses of DAF, MCP, CR1, CR2, and Crry/p65 homologous in other species should continue to be instructive in this regard and will allow the further refinement of evolutionary models. In this regard, a Crry/p65 homologue in rat has recently been identified and partially sequenced (47, 48). This rat protein also exhibits complement regulatory activity. Based on the common SCR structures and biochemical activities, it is likely that DAF and MCP as well as Crry/p65 share a more distant evolutionary progenitor. Whatever the results of those future analyses, it is already apparent that the repetitive SCR containing structure of the RCA genes has allowed a unique evolutionary process to proceed.

Finally, we believe that mouse Crry/p65 can serve as an effective model for the biologic activities of DAF and MCP. In addition to the protection of normal cells from inadvertent complement pathway activation, several other biologic roles have been proposed for the normal expression or, in some cases, the overexpression complement regulatory proteins. These roles include the protection of placenta and fetus from complement fixing "alloantibody" (49), sperm from anti-sperm antibodies and complement in the female reproductive tract (50), tumors from antitumor antibody (26), and normal cells from NK activity (51). Analysis of the *in vivo* role of Crry/p65 in various mouse models should further increase our understanding of the biologic roles of membrane complement regulatory proteins.



**Figure 6.** Exon-intron organization of RCA genes encoding mouse and human membrane proteins. *Bracket* indicates four SCR-containing motif present in mouse *Crry* and *Cr2* in addition to human CR1 and CR2. DAF and MCP lack the motif. Ligands that bind within the motifs are shown. The site of alternative splicing within *Cr2* gene between exons 6b and 7,8 is shown by the Y.

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