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Immunology

Identification of murine complement receptor type 2

(complement component C3d receptor/Epstein–Barr virus receptor/nucleotide sequence/gene evolution)

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ABSTRACT **A rabbit antiserum reactive with the human complement component C3d/Epstein–Barr virus receptor (complement receptor type 2, CR2) immunoprecipitates a M_r 155,000 murine B-cell surface antigen. The apparent molecular weight and cellular distribution of this murine antigen are similar to those of human CR2. Cells expressing the murine protein bind sheep erythrocytes coated with antibody and murine C1-C3d but do not bind Epstein–Barr virus at all. The monospecific antiserum to human CR2 together with goat $F(ab')_2$ anti-rabbit IgG blocks attachment of the C3d-coated erythrocytes to receptor-bearing murine B lymphocytes. To further characterize murine CR2, a λgt11 library from the murine late pre-B-cell line 70Z/3 was screened with human CR2 cDNA. A partial cDNA clone of 3.5 kilobases with 79% amino acid sequence identity to human CR2 in the unique intracytoplasmic region and 63% identity to the sixth human CR2 repeat was obtained. Blot hybridization with the murine cDNA clone identified an RNA species of ≈4.7 kilobases, similar in size to human CR2 mRNA, from a murine B-cell line but not from a murine T-cell line.**

A significant role for the third component of complement, C3, as an accessory signal in induction of the humoral immune response has been postulated on the basis of observations demonstrating deficient antibody production in mice depleted of complement *in vivo* (1) as well as in animals (guinea pigs, dogs) that are genetically deficient in C3 (2, 3). A further role for the d fragment of C3 (C3d) has been suggested by studies correlating impaired humoral immune response, but not impaired phagocytic function, with serotypes of *Pneumococcus* that inhibit the degradation of C3b to C3d on their surface capsule (4).

The human receptor for C3d, complement receptor type 2 (CR2) is a M_r 145,000 membrane glycoprotein that is also the receptor for Epstein–Barr virus (EBV) (reviewed in ref. 5). CR2 is part of a multigene family of complement regulatory or receptor proteins (C4-binding protein, factor H, decay-accelerating factor, membrane cofactor protein, and CR1) that map to chromosome 1, band 1q32, and that consist primarily of ≈60 amino acid repeat units sharing specificity for C3b/C4b (reviewed in refs. 6 and 7), with the exception of CR2, which exhibits primary specificity for C3d. The molecular structure of CR2 has recently been determined (8, 9). Experiments suggest that triggering of CR2 with crosslinked ligand (C3d), monoclonal antibodies, or EBV (including nontransforming strains or UV-inactivated virus), in conjunction with T-cell factors, may stimulate B-cell activation (5). Divalent antibodies to B-cell surface membrane IgM or IgD (sIgM or sIgD) have been reported to cap ligand-loaded CR2 (10), and antibodies to sIgM induce phosphorylation of CR2 (11). Attachment of immune complexes bearing antigen and C3d to the B-cell surface could theoretically crosslink sIg and CR2 (to themselves or to each other). Recent studies (ref. 12 and unpublished observation) support the notion that this event may amplify the signal delivered through sIg or, alternatively, may provide two independent signals that, when delivered concomitantly, produce synergistic and T-cell-independent B-cell activation.

Murine B-lymphocyte receptors for C3b and C3d have been defined by ligand binding (13–15), as monospecific reagents identifying the distinct receptors have not been available. A recently described monoclonal antibody reacts with a murine membrane protein of M_r 190,000 with properties that suggest it is the homolog of human CR1 (16). A previously described rabbit antiserum directed to CR1 immunoprecipitated a protein of M_r ≈65,000 (p65) from murine hematopoietic cells and fibroblasts (17). B-cell complement receptors have been reported to exhibit delayed development in neonatal mice (18) and to be deficient in mice with X chromosome-linked immunodeficiency, which fail to mount a vigorous humoral response, especially to T-independent antigens (19, 20). The precise receptor(s), however, have not been identified. Altered human complement receptor expression is associated with rheumatologic disease (21, 22), yet similar abnormalities (23) have not been detailed in inbred mice manifesting lupus-like illnesses and characterized by polyclonal B-cell proliferation (24, 25)—presumably because of a lack of monospecific reagents.

In this report, the murine homolog of CR2, which is not an EBV receptor, is identified as a M_r 155,000 membrane protein and a partial cDNA clone is characterized.‡

MATERIALS AND METHODS

Preparation of Rabbit Anti-Human CR2. Purified human CR2 (26) suspended in 0.1% Nonidet P-40 was injected subcutaneously into rabbits in complete Freund's adjuvant (50 μg) for primary immunization and in incomplete Freund's adjuvant (15 μg) for booster injections. The antiserum immunoprecipitated a M_r 145,000 protein (Fig. 1*a*, lanes 1) from detergent lysates of human CR2-bearing cells.

Mice. Swiss nude (*nu/nu*) mice were supplied by Taconic Farms (Germantown, NY). BALB/c, DBA/2, CBA/J, and C57B10.D2/OSN(C5-deficient) mice were supplied by The Jackson Laboratories.

Isolation of Murine B and T Lymphocytes. Murine splenic lymphocytes and monocytes were isolated by gradient centrifugation. B cells and monocytes were separated from T cells by passage through a nylon-wool column (27) or by panning (28) on plates coated with anti-mouse immunoglobulin followed by adherence on plastic.

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Abbreviations: C*n*, complement component *n*; C3b and C3d, fragments of C3; CR1, complement receptor type 1 (C3b/C4b receptor); CR2, complement receptor type 2 (C3d receptor); EA, sheep erythrocyte(s) sensitized with rabbit IgM (19S antibody); $EAC3d^{mo}$, EA coated with murine C1–C3d; EBV, Epstein–Barr virus; FITC, fluorescein isothiocyanate; PRS, preimmune rabbit serum; sIg, surface immunoglobulin.

‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04153).

Immunoprecipitation. Splenic lymphocytes (5×10^7) were radiolabeled and immunoprecipitated as described (29). Solubilized ^{125}I-labeled proteins were precleared twice with nonimmune rabbit serum and specifically immunoprecipitated by addition of 50 μl of rabbit anti-human CR2 or preimmune rabbit serum (PRS). $NaDodSO_4$/7.5% PAGE was performed on the reduced protein.

Immunoblotting. Immunoblot analysis (29) of murine splenocytes used rabbit anti-human CR2 or PRS diluted 1:100.

Cell Lines. Human lines used included Raji (Burkitt lymphoma), K562 (erythroleukemia) and CEM (T cell). The mouse cell lines are listed in Table 1 and have been described (30).

EBV Binding Assays. Purification of EBV, coupling to fluorescein isothiocyanate (FITC), and EBV binding assays were as described (31).

Flow Cytometry. Cells ($\approx 10^6$) were incubated with 200 μl of rabbit anti-human CR2 or PRS (diluted 1:100) followed by FITC-F(ab')$_2$ goat anti-rabbit IgG (Cooper Biomedical, Malvern, PA), or FITC-EBV and analyzed by flow cytometry (26).

Rosette Assays. Sheep erythrocytes sensitized with rabbit IgM (EA) were purchased (Diamedix Laboratories, Miami). EA coated with guinea pig C1–human C3d were obtained as a special order (Diamedix). EA coated with murine C1–C3d ($EAC3d^{mo}$) were prepared essentially as described (15), employing C5-deficient mouse serum. Rosette assays were performed as described (15) by mixing $EAC3d^{mo}$ with nucleated cells that had been preincubated with appropriate antibodies (rabbit anti-human CR2 or normal rabbit serum diluted 1:50 or goat F(ab')$_2$ anti-rabbit IgG (Cooper Biomedical) diluted 1:25. Cells with >3 erythrocytes bound were scored positive. For each sample 300 nucleated cells in random fields were counted by two observers.

Isolation of a cDNA clone. Oligonucleotides based on protein (26) and partial cDNA (32) sequence were synthesized and pooled to screen a λgt10 library from the human B-cell IB4 (33) and a λgt11 library from the human B-cell line JY (34). Two overlapping human cDNA clones were used to screen a λgt11 library from the murine late pre-B-cell line 70Z/3 (35). Under stringent conditions one plaque hybridized in duplicate to the probes. Phage DNA was prepared and a single 3.5-kilobase (kb) cDNA excised from the λgt11 vector with *Eco*RI was subcloned into pUC13. The murine cDNA was digested with various restriction enzymes to generate a map and to give smaller fragments for sequence analysis (36).

Blot Analysis of CR2 Transcripts. Cellular RNA was isolated (37), electrophoresed (30 μg per lane) in a 2.2 M formaldehyde/1% agarose gel, blotted onto nitrocellulose, and hybridized with nick-translated ^{32}P-labeled murine CR2 or actin (38) cDNA ($1–5 \times 10^8$ cpm/μg).

RESULTS

Rabbit Anti-Human CR2 Recognizes a M_r 155,000 Murine B-Cell Surface Antigen. To determine whether immunological crossreactivity between human and murine CR2 was conserved, a rabbit antiserum prepared against affinity-purified human CR2 was used to immunoprecipitate detergent lysates of ^{125}I-labeled murine splenic lymphocytes. Immunoprecipitation of lysates from splenic lymphocytes of athymic nude mice (pure B-cell population) with rabbit anti-human CR2, but not with PRS, revealed a single band at M_r 155,000 upon analysis by $NaDodSO_4$/PAGE (Fig. 1*a*, lanes 2). Comparison of the mouse protein to that observed upon immunoprecipitation of human CR2 from Raji cells (Fig. 1*a*, lanes 1) revealed that the murine molecule was slightly larger than the M_r ≈145,000 human receptor. Analysis of splenic lymphocytes from BALB/c mice before (Fig. 1*a*, lanes 3) and after

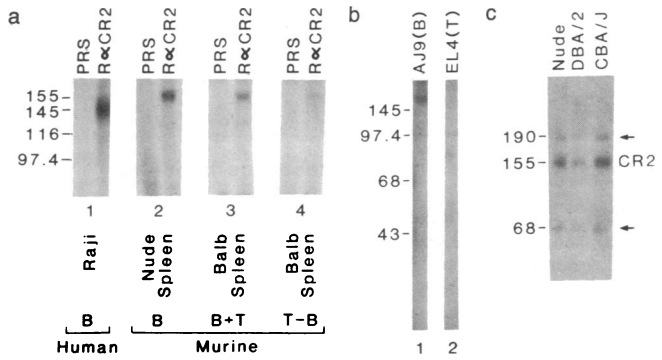

FIG. 1. Rabbit anti-human CR2 (RαCR2) recognizes a M_r 155,000 murine B-cell antigen. (*a*) Immunoprecipitation of lysates of ^{125}I-labeled cells with PRS and with RαCR2. Lanes: 1, Raji (human B-cell line); 2, nude mouse splenic lymphocytes (pure B-cell population); 3, BALB/c splenic lymphocytes (equal numbers of B and T cells); 4, BALB/c splenic lymphocytes specifically depleted of B cells. (*b*) Immunoprecipitation with RαCR2. Lanes: 1, murine B-cell line AJ9; 2, murine T-cell line EL4. (*c*) Prolonged exposure of an immunoblot of proteins of splenic lymphocytes from nude, DBA/2, and CBA/J mice. Faint bands indicated by arrows appear at $M_r \approx$ 190,000 and 68,000 in addition to the distinct band at M_r 155,000, observed upon immunoprecipitation. Numbers at left indicate $M_r \times 10^{-3}$.

(lanes 4) removal of B lymphocytes demonstrated that the crossreactive murine antigen resided in the B-cell fraction. In addition, a murine B-cell line of mature phenotype, AJ9, revealed the M_r 155,000 receptor, whereas no antigen was present on a murine T-cell line, EL-4 (Fig. 1*b*). Immunoblot analysis of splenic lymphocytes from three distinct mouse strains (Fig. 1*c*) revealed a predominant M_r 155,000 band, but upon prolonged exposure, faint bands could also be detected at $M_r \approx$190,000 and 68,000, similar to the positions expected for murine CR1 (M_r 190,000) and p65 [possibly the homolog of membrane cofactor protein (6)] suggesting that, when completely denatured, homologous proteins may also be detected with this antiserum.

Cellular Distribution of the Crossreactive Murine Protein. Expression of human CR2 on B cells as well as on certain T-cell lines has been established by flow cytometry, immunoprecipitation, and protein purification. During human B-

Table 1. Expression of CR2 on a panel of murine cell lines

Cell line	Phenotype	% positive*
300-19	Early pre-B	3
54.3	Classic pre-B	1
PD31	Late pre-B	3
70Z/3	Late pre-/early B	30
A20	Mature B	44
L10A/J	Mature B	82
AJ9	Mature B	84
BCL-1	Mature B	8
M1241	Mature B	28
S194	Myeloma	1
MOPC 104E	Myeloma	2
EL4	Thymoma	7
BW5147	Thymoma	1
YAC-1	T	6
P388	Monocyte/macrophage	12
P815	Mastocytoma	1

*Calculated from immunofluorescence histograms generated by analytical flow cytometry as the ratio of the area under the curve with rabbit anti-human CR2 (specific antibody) that did not intersect the curve with PRS (irrelevant antibody) (autofluorescence curve) to the total area under the curve with specific antibody. These values therefore represent minimum percentages; e.g., inspection of Fig. 2 suggests that 100% of A20 and L10A/J cells are positive. Each value represents an average of 1–7 determinations.

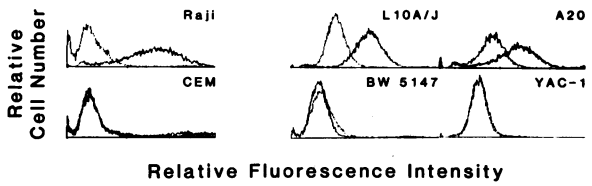

FIG. 2. Flow cytometry with rabbit anti-human CR2. Human (Raji) and murine (L10A/J and A20) B-cell lines express surface CR2, whereas human (CEM) and murine (BW5147 and YAC-1) T-cell lines do not, as shown by coincidence of the autofluorescence curves with the immunofluorescence curves. Relative fluorescence intensity is shown on a logarithmic scale.

cell ontogeny CR2 is absent on the early pre-B cell, appears as the B cell matures into the virgin lymphocyte, and disappears with terminal differentiation into the plasma cell (39, 40). Eleven murine cell lines representing distinct stages of B-cell differentiation were examined (Table 1) for expression of the crossreactive antigen. Comparative staining of a mature human B-cell line (Raji) and two mature murine B-cell lines (L10AJ and A20) are presented (Fig. 2). Whereas three murine early pre-B-cell lines and two myeloma cell lines (representing terminally differentiated B cells) did not express this murine receptor (Table 1), one late pre-B-cell line, 70Z/3, and four mature B-cell lines, A20, M1241, L10AJ, and AJ9, had detectable antigen; one mature B-cell line, BCL-1, did not. These results parallel previous observations with human CR2. Three murine T-cell lines and a mastocytoma did not express this antigen (Table 1). A murine macrophage line expressed low antigen levels, consistent with possible Fc receptor binding.

Rabbit Anti-Human CR2 Inhibits Binding of EAC3d^{mo} to Its Receptor. To determine whether the murine antigen functioned as a C3d receptor, EAC3d^{mo} were prepared. EAC3d^{mo} avidly bound (>3 per cell) both human and mouse cells bearing CR2 as assessed by staining with either the antiserum or monoclonal antibodies (39, 40) and erythrocyte adherence correlated quantitatively with the predicted number of cellular receptors on four human cell lines (data not shown). As previously reported (14), EA coated with human C3d bound less well to murine than to human B cells (e.g., only 10–20% of AJ9 cells bound >3 EA bearing human C3d; data not shown). When EAC3d^{mo} were mixed with Raji cells [which do not express detectable CR1 (31)] alone or following preincubation with irrelevant antiserum (Table 2), >97% of the cells bound EAC3d^{mo}, whereas <3% of the human receptor-negative K562 cells did so. Under identical conditions 84–93% of murine AJ9 cells formed rosettes, whereas murine T cells (YAC-1) did not. Fewer EAC3d^{mo} bound to AJ9 cells than to Raji cells, consistent with a lower receptor density. Preincubation of Raji with rabbit anti-human CR2 alone eliminated binding of EAC3d^{mo}, whereas attachment to AJ9 was minimally reduced (to 82%). However, binding of the antiserum to AJ9 followed by addition of goat anti-rabbit IgG abolished binding of EAC3d^{mo}. These results suggest that, although murine C3d can serve as ligand for both the human and murine receptors, greater specificity of the rabbit antiserum for human CR2 results either in production of higher-affinity antibodies or in recognition of epitopes at or near the ligand binding site of human but not of murine CR2, thereby requiring additional crosslinking to inhibit rosette formation.

Table 2. Rabbit anti-human CR2 (RαCR2) blocks binding of EAC3d^{mo} to human and murine CR2-bearing cell lines

Cell line	% rosette formation*					
	No serum	NRS	GαR	NRS + GαR	RαCR2	RαCR2 + GαR
Human						
Raji	>97	>97	>97	>97	<3	<3
K562	<3	<3	<3	<3	<3	<3
Murine						
AJ9	84	93	85	89	82	<3
YAC-1	<3	<3	<3	<3	<3	<3

NRS, normal rabbit serum; GαR, goat F(ab')$_2$ anti-rabbit IgG.
*Nucleated cells (300 per determination) were scored positive when >3 erythrocytes bound per cell.

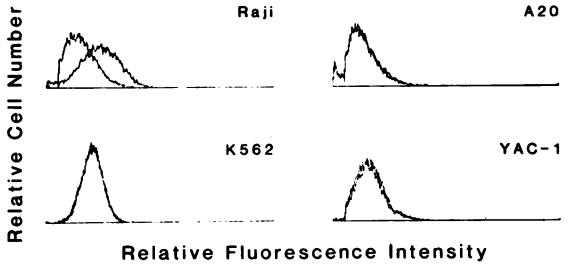

FIG. 3. Flow cytometry with FITC-EBV. FITC-EBV bound to the human CR2$^+$ B-cell line Raji but not to the human CR2$^-$ erythroleukemia cell line K562, the murine CR2$^+$ B-cell line A20 (see Fig. 2), or the murine CR2$^-$ T-cell line YAC-1. The scale for relative fluorescence intensity is logarithmic.

Murine B Cells Do Not Bind EBV. EBV can infect and transform B lymphocytes from humans and from certain New World primates. The specific events that block infection/transformation of other species have not been systematically examined. To assess whether EBV could bind CR2-bearing murine B cells, FITC-EBV was incubated with the murine receptor-bearing B-cell line A20, as well as with the murine

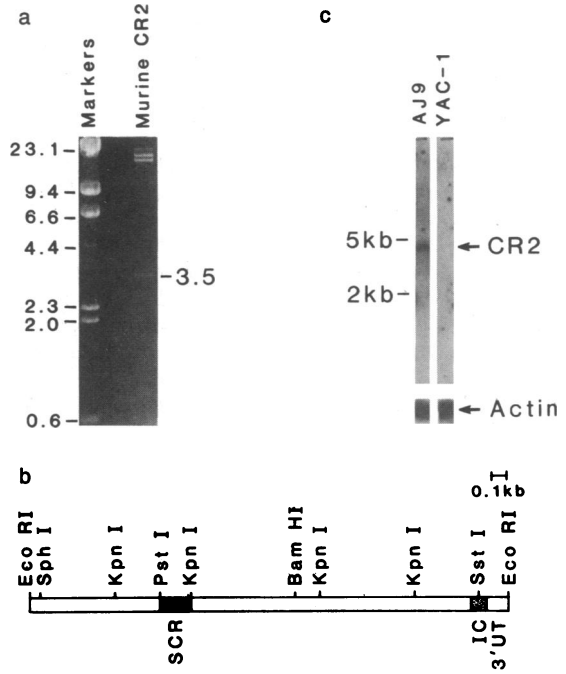

FIG. 4. Characterization of a partial cDNA clone encoding murine CR2. (*a*) *Eco*RI-digested, ethidium bromide-stained fragments of murine CR2 cDNA in λgt11. Marker sizes (kb) are indicated at left, and estimated size of the insert is indicated at right. (*b*) Map of a partial cDNA for murine CR2 generated with the restriction enzymes indicated. Positions of the short consensus repeat (SCR) described in Fig. 5*a* (black), intracytoplasmic (IC) tail (stippling), and 3′ untranslated (3′UT) portion are indicated. (*c*) Blot hybridization of 30 μg of total RNA from murine B (AJ9) and T (YAC-1) cell lines probed with nick-translated murine CR2 or actin probes. Migration positions of 28S (5 kb) and 18S (2 kb) RNA are indicated at left.

T-cell line YAC-1, which does not express this protein (Fig. 2). Neither A20 nor YAC-1 bound FITC-EBV, whereas under identical conditions the human B-cell line Raji bound FITC-EBV but the CR2-negative human line K562 did not (Fig. 3).

Characterization of a cDNA Clone Encoding Murine CR2. A λgt11 cDNA clone that hybridized to human CR2 probes under stringent conditions was digested with *Eco*RI. A single 3.5-kb insert, identified by agarose gel electrophoresis (Fig. 4*a*), was extracted and subcloned into pUC13, and a restriction enzyme map was generated (Fig. 4*b*). Blot hybridization analysis of total RNA (Fig. 4*c*) with either the intact cDNA or a 3′ *Eco*RI–*Sst* I subfragment as probe revealed that under stringent conditions the murine clone hybridized to a single RNA species of ≈4.7 kb from the murine B-cell line AJ9, but no hybridization was detected with RNA from the murine T-cell line YAC-1. At minimally lowered stringency, with the intact cDNA as probe, RNA species of approximately 9 kb and 2 kb could also be detected, which would be consistent with the predicted mRNAs for murine CR1 and decay-accelerating factor or potentially membrane cofactor protein [although the human message is larger (6)] or as yet unidentified family members.

The insert was subcloned into M13 for sequence analysis, which revealed a structure consisting of tandem repeats with highly conserved amino acids in positions found in other human and murine complement regulatory-locus proteins (8, 41) (Fig. 5*a*). Stringent hybridization of selected subfragments of human CR2 to the murine clone and sequence data suggested approximate alignment of the two molecules. Murine nucleotide sequence corresponding to the eleventh human tandem repeat (8), which may represent an allelic polymorphism (9), was not detected. Comparison of the sixth short consensus repeat of human CR2 and a murine consensus repeat (SCR in Fig. 4*b*) demonstrated an overall amino acid identity of 63% (75% nucleotide identity) with complete identity of highly conserved elements. One potential N-linked glycosylation site was conserved and an additional site uncovered in the murine sequence (Fig. 5*a*).

The predicted length of the cytoplasmic tail of mouse CR2 was 35 amino acids, similar to human CR2 with 34 amino acids, whereas human CR1 contained 43 amino acids in the cytoplasmic region (Fig. 5*b*). The predicted amino acid sequence of the cytoplasmic tail of murine CR2 was 79% identical to that of human CR2 but only 28% identical to that of human CR1 and included eight of nine potential phosphorylation sites, suggesting marked conservation of function in this domain. The murine cDNA contained a modified polyadenylylation signal and poly(A) tail. The 3′ untranslated portion of this clone was significantly shorter (157 nucleotides) than in human counterpart [789 (8) or 771 (9) nucleotides], suggesting greater genetic divergence in this region, although additional 3′ sequences not identified in this cDNA could also account for observed differences.

DISCUSSION

A rabbit antiserum prepared by immunization with purified human CR2 that had been suspended in mild detergent to

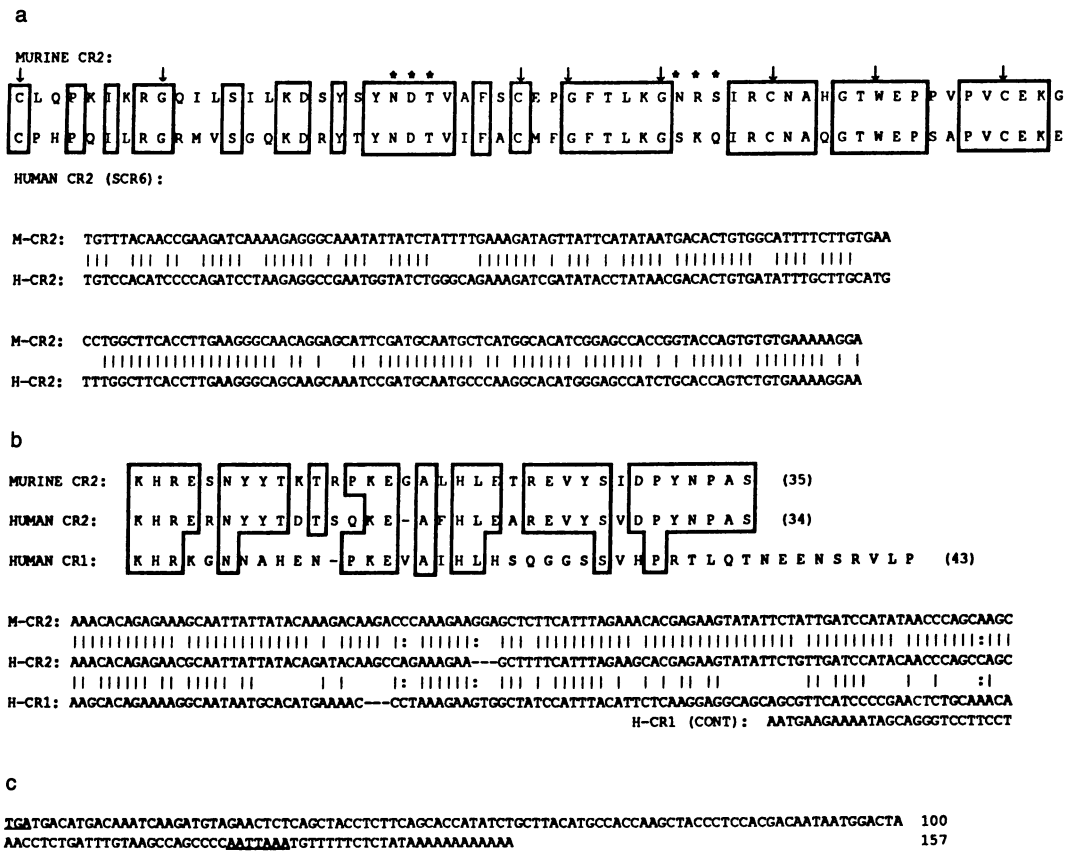

FIG. 5. Comparison of human (8, 9) and murine CR2. (*a*) The ≈60 amino acid consensus repeat sequence structure is conserved in murine CR2. (*b*) The cytoplasmic domain of murine CR2 (35 amino acids) is homologous to human CR2 and human CR1. (*c*) The 3′ untranslated end of murine CR2 is shorter than that of human CR2. Amino acids are shown in standard one-letter symbolism. Boxes indicate identity of the predicted amino acids. Arrows indicate highly conserved positions found among all members of the human complement regulatory-locus gene family (8). Stars indicate potential N-linked glycosylation sites. Vertical lines indicate exact nucleotide identity between human (H-) and murine (M-) CR2. Double dots indicate exact nucleotide identity between M-CR2 and H-CR1 not present in H-CR2. The stop codon and polyadenylylation signal are underlined. Human CR2 sequence derived from Raji (8) is presented where differences are identified.

conserve secondary structure precipitated a M_r 155,000 membrane protein from murine B cells but not from murine T cells. The antiserum recognized murine lymphocytes of a mature B-cell phenotype, consistent with the established ontogeny of human CR2. The antiserum blocked attachment of ligand to the murine receptor only in the presence of second antibody, suggesting that although the homologous receptor was recognized, either the affinity for murine CR2 was low or epitopes mediating binding of murine C3d to the membrane were not recognized. A 3.5-kb mouse cDNA clone was obtained from a murine λgt11 library screened with human CR2 probes. Sequence data from this clone revealed a structure with homologous repeat units and a carboxyl-terminal intracytoplasmic domain with 79% amino acid sequence identity to human CR2, suggesting significant conservation of function. Interestingly, although the murine message appeared to be approximately the same size as that of human CR2, the mouse protein was larger estimated by $NaDodSO_4$/PAGE, raising the possibility that additional glycosylation sites were present or were utilized and potentially accounting for some of the differential ligand specificities. One such additional glycosylation site was identified.

Cloning of human CR2 will permit identification of amino acid sequences important for the attachment of C3d and EBV. Comparison with murine CR2, with its distinct pattern of ligand binding, may prove helpful in predicting potential binding sites, and construction and expression of chimeric human–mouse receptor(s) may prove useful in clarifying binding and internalization events. Similarly, comparison of homologous sequences shared by the human and murine ligands of CR2 may be helpful in predicting salient attachment sites on these molecules.

The precise functional role(s) of CR2 in B-cell activation remains to be determined. Ligand-loaded CR2 and human sIgM or sIgD cocap upon stimulation with divalent monoclonal antibodies (13). In the mouse, sIgD has been reported to cocap with CR1 and to a lesser extent with CR2, whereas sIgM has not been reported to cocap with these receptors as identified with ligand-coated erythrocytes (42). Investigation of pertinent human and murine capping mechanisms should now become possible. Functional studies on the role of CR2 in signal transduction in normal murine B cells and functional as well as genetic studies in inbred mouse strains with autoimmune diseases, such as lupus, characterized by B-cell dysregulation and polyclonal proliferation may expand our understanding of the importance of this molecule *in vivo*.

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