Identification of Mutations in Rat CD59 That Increase the Complement Regulatory Activity†

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ABSTRACT: Formation of the membrane attack complex (MAC) of complement on host cells is inhibited by the glycosylphosphatidylinositol- (GPI-) anchored glycoprotein CD59. Published data on the active site of human CD59 are confusing. To clarify these data, we set out to elucidate the active site of a nonprimate CD59 molecule by site-directed mutagenesis. We also undertook to investigate a region of potential species selectivity, and to this end rat CD59 was chosen for all mutations. Our investigations confirmed the proposal that the active site of CD59 is the major hydrophobic groove, with mutations Y36A, W40A, and L54A ablating complement inhibitory function of CD59. Other mutations reducing the function of rat CD59 were I56E, D24A, and D24R. Importantly, mutations at one residue increased the function of rat CD59. The K48E mutation significantly increased function against human rat or rabbit serum, whereas the K48A mutation increased function against human serum alone. A similar mutation in human CD59 (N48E) had no effect on activity against human or rat serum but completely abolished all activity against rabbit serum. These findings suggest that the α-helix of human CD59, adjacent to the hydrophobic groove, influences the interaction between human CD59 and rabbit C8, C9, or both.

The major inhibitor of the complement membrane attack complex (MAC)[1] is CD59, an 18−20 kDa glycosylphosphatidylinositol- (GPI-) linked glycoprotein, expressed on a wide range of cell types of both hemopoietic and nonhemopoietic origins (*1*). To date, analogues of CD59 have been characterized and cloned from a variety of nonprimate species, including rat, mouse, pig, and rabbit, and have been shown to function across species barriers, albeit to varying degrees (*2*−*6*). The complement inhibitory activity of CD59 lies in its ability to bind to the α-chain of C8 in the C5b-8 complex, and also to bind to the b domain of C9, thus preventing the unfolding and polymerization of C9 (*7*−*9*). The conservation of function across species suggests that the active site responsible for binding C8/C9 in CD59 is conserved across species. The slight differences in inhibitory function between different species sera have been termed the species selectivity of CD59. These differences are likely to be due to the effect of nonconserved residues in close proximity to the active site.

Several groups have undertaken domain swapping and site-directed mutagenesis studies to elucidate the active site of human CD59 and the residues involved in its species selectivity (*5*, *10*−*14*). The confusing data produced by these studies have implicated a surface region centered on W40 as a putative active site. In the published NMR structures of human CD59, W40 lies at the base of a hydrophobic groove formed by residues Y36, C39, W40, and L54, with a ridge of hydrophilic residues (K41, H44, R53, and R55) on one side (*15*, *16*). Several of these residues were implicated as components of the active site by mutational studies, suggesting that this was the active site of CD59 (*10*−*12*). This suggestion was supported by the demonstration that the binding of function-blocking monoclonal antibodies was reduced or abolished by mutation of residues in and around this hydrophobic groove (*10*, *11*).

Two other residues distant from the hydrophobic groove have also been shown to influence function. A D24R mutation in human CD59 completely abrogated the function of the molecule (*11*). Residue D24 is part of a highly conserved extruding loop domain on the opposite face of CD59 to the groove. This loop comprises residues 20−24 and was predicted on the basis of its conservation between species to be involved in function (*2*). The functional role of this 20−24 loop has never properly been assessed. The other residue shown to influence function is Y61. A series of mutations in Y61 all reduced inhibitory function against human serum, but to differing degrees (*10*). This residue is part of a triple β-sheet in CD59, distant from the hydrophobic groove. Residues D24 and Y61 are physically distant from each other and thus are not components of a second active site.

Attempts to identify residues involved in species selectivity have focused on human CD59 and have compared function with rat and rabbit analogues (*5*, *13*, *14*). Human CD59 inhibits rat and rabbit serum poorly, whereas rabbit CD59 inhibits human serum poorly and rat CD59 efficiently inhibits

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[1] Abbreviations: BSA, bovine serum albumin; FCS, fetal calf serum; GPI, glycosylphosphatidylinositol; mAb, monoclonal antibody; MAC, membrane attack complex; PBS, phosphate-buffered saline; VBS, veronal buffered saline.

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all these sera. Chimeras between rat and human CD59 molecules have implicated two separate regions in the species selectivity of CD59, one between human residues 40 and 47 and the second between human residues 47 and 66 (*13*). Individual and multiple mutations of this region identified residues F47, T51, R55, and K65 in the α-helix of human CD59 to be responsible for the poor inhibition of rat serum by human CD59 (*14*). A separate study involving the substitution of regions between human and rabbit CD59 has implicated human residues 42−58 in species selectivity (*5*).

The primary purpose of this study was to confirm that the proposed active site of human CD59 was conserved in other species and to determine the residues responsible for species selectivity between rodent and nonrodent CD59s. To address these issues, a series of mutations were generated in rat CD59. Alanine substitutions were made in residues in and around the hydrophobic groove and α-helix to assess their effect on the functional activity of the molecule. Also a number of residues in the α-helix were mutated to their murine counterparts in order to assess their influence on the species selectivity of the molecule. These mutant CD59s were expressed at similar levels on Chinese hamster ovary (CHO) cells and their function was tested against rat, human and rabbit sera.

EXPERIMENTAL PROCEEDURES

Molecular Biology. All general reagents were from Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. Oligonucleotide primers were from Gibco−BRL (Paisley, U.K.). Taq polymerase and buffers were from Pharmacia (Milton Keynes, U.K.) and dNTPs were from Bioline (London, U.K.). All restriction enzymes were from Amersham International (Little Chalfont, U.K.). GeneClean II DNA purification kit was from Anachem (Luton, U.K.) and plasmid purification kits were from Qiagen (Dorking, U.K.). The expression vector pDR2ΔEF1α was a gift from Dr. I. Anegon (INSERM U437, Nantes, France) (*17*) and contains the hygromycin resistance gene, allowing the selection of stable colonies, and the powerful polypeptide chain elongation factor 1α promoter.

Tissues, Cells, and Sera. Rabbit and rat sera were obtained fresh from the animal facility of the University of Wales College of Medicine. Human serum was obtained fresh from healthy volunteers. All serum was stored at −70 °C. Rat erythrocytes were obtained fresh from the same facility and collected into 20 mM EDTA prior to processing for flow cytometry.

CHO-K1 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were propagated in RPMI supplemented with 10% fetal calf serum, glutamine, pyruvate, and penicillin/streptomycin. All tissue culture reagents were obtained from Gibco−BRL (Paisley, U.K.).

Antibodies. Monoclonal antibodies (mAbs) and polyclonal antiserum against rat CD59 were generated in-house as described previously (*18*). Mouse monoclonal anti-myc was obtained from Invitrogen (Groningen, Netherlands). Phycoerythrin-conjugated secondary antibodies against mouse Ig were obtained from Dako Ltd. (Bucks, U.K.).

Generation of Rat CD59 myc Tag Construct. A c-myc epitope was inserted between the first and second residues of the mature rat CD59 protein by a two-stage PCR process. Full-length rat CD59 cDNA (*2*) was used as the template. Primers R59V (GGTTCTAGAGTGGACCAGCACAATGAGAG) and R59VSL (AGATCCTCTTCTGAGATGAGTTTTTGTTCGAGGCTAACACCTGTGGAACAG) were used to amplify by PCR a 118 bp product that consisted of an *Xba*I restriction site, the first 82 bp of rat CD59 sequence up to and including residue L1, followed by 29 bp of the c-myc sequence. Primer R59T (GTGGGATCCAGGCATCGGGAGCTTAGAG) and R59RCY (CAAAAACTCATCTCAGAAGAGGATCTGAGATGCTACAACTGTTTAGACCC) were used to amplify by PCR a 372 bp product that consisted of 27 bp of c-myc cDNA followed by 336 bp of rat CD59 sequence from residue R2 onward, ending in a *Bam*HI restriction site. All PCRs were performed using the following conditions: 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min.

The two PCR products were purified from a 1% agarose gel by GeneClean (Anachem), and the concentration of DNA was measured by A_{260}. The purified products contained overlapping regions of 26 bp and were mixed in equimolar ratios and used as a template for PCR amplification of the full length construct with the two outside primers R59V and R59T (15 cycles of 94 °C for 30 s and 72 °C for 1 min). The full-length 455 bp product was digested with *Xba*I and *Bam*HI, purified by GeneClean (Anachem), and ligated into the pDR2ΔEF1α expression vector (*17*). The resulting colonies were screened and sequenced, and a colony (myc6) containing no errors was chosen for mutagenesis.

Site-Directed Mutagenesis by Two-Stage PCR. Mutations were generated by two-stage PCR as described above, using two complementary 21-mer primers containing the desired mutation and primers R59V and R59T as the outside primers. The myc-tagged rat CD59 (myc6) was used as the template. All these PCRs were performed with Taq DNA polymerase (Promega) under the following PCR conditions: 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min.

Double mutants were produced by using the full-length PCR product from one round of mutagenesis as the template for two-stage PCR of the second mutation.

The full-length PCR products were purified by GeneClean (Anachem), digested with *Xba*I and *Bam*HI for 90 min at 37 °C, and ligated into the pDR2ΔEF1α expression vector. Colonies were screened and plasmids were sequenced to ensure fidelity and check for the desired mutation.

Expression of Rat CD59 Mutants in CHO Cells. Plasmids containing the mutated rat CD59 constructs were transfected into CHO-K1 cells by lipofection according to the manufacturer's protocol (Lipofectamine Plus; Gibco−BRL). After lipofection, cells were returned to sterile flasks and cultured for 24 h in 10 mL of fresh RPMI containing 10% FCS. Cells were washed once in sterile 0.9% NaCl and resuspended in selection medium (RPMI containing 10% FCS and 0.4 mg/mL hygromycin B). Selection medium was changed every 2 days for approximately 2 weeks, by which time all the nontransfected control cells had died. Transfected cells were then maintained in RPMI containing 10% FCS and 0.1 mg/mL hygromycin B (Gibco).

Analysis of Binding of Monoclonal Antibodies by Flow Cytometry. Cells were trypsinized, washed once in 0.9%NaCl, and resuspended at 10^6/mL in PBS/1% BSA. Expressing cells were then assayed for binding of the four mouse anti-rat

CD59 mAbs, a mouse anti-c-myc mAb (Invitrogen) and a rabbit anti-rat CD59 polyclonal antiserum (in house). Cells were incubated at 4 °C for 30 min with either 10 μg/mL monoclonal antibody or a 1:5 dilution of the polyclonal antiserum. Cells were then washed 3× in PBS/1% BSA and incubated at 4 °C for a further 30 min with a 1:400 dilution of an appropriate RPE-conjugated secondary antibody. Cells were then washed 3× in PBS/1% BSA, and fluorescence was measured with a FACScalibur flow cytometer (Becton-Dickinson).

Functional Analysis of Rat CD59 Mutants. Transfected cells and rat cell lines were assayed for inhibition of complement lysis by the calcein release assay. Cells were seeded at 10^5/well in 24 well plates, 24 h prior to assaying. Cells were washed once in 0.9% NaCl and overlaid with 250 μL of calcein-AM (Molecular Probes, Oregon) at 2 μg/mL in Dulbecco's modified Eagle's medium (DMEM)/10% FCS. After incubation at 37 °C for 30 min, the cells were washed once in PBS and overlaid with 250 μL of heat-treated polyclonal anti-CHO antiserum diluted 1/5 in VBS/1% BSA. Cells were then incubated for 15 min at 4 °C, before being washed once in PBS. Cells were then overlaid with 250 μL of fresh serum diluted in VBS/1% BSA. Cells were incubated at 37 °C for an optimal time depending on the serum source. The supernatant was then transferred to a flat-bottomed 96 well plate for analysis. The cells were overlaid with 0.1% Triton X-100 and incubated at room temperature for 10 min to lyse remaining cells. The supernatant was transferred to a separate 96 well plate for analysis. Levels of calcein released from cells by serum and Triton X-100 were measured by the WellFluor system (Denley) with excitation at 485 nm and emission at 530 nm.

Percentage lysis by serum was calculated as follows: [(calcein released by complement)/(calcein released by complement + calcein released by detergent)] × 100.

RESULTS

Construction and Expression of Rat CD59 Mutants. The expression and correct folding of the wild-type and mutant CD59s in CHO cells was determined by flow cytometry with an anti-myc monoclonal antibody and an anti-rat CD59 polyclonal antiserum. The levels of antibody binding are shown in Table 1.

For every mutant, both anti-myc mAb and polyclonal anti-rat CD59 bound well, demonstrating expression of all mutant CD59s with only slight variation in levels of expression. These data contrast with previous human CD59 mutagenesis studies where several mutants have failed to be properly expressed, presumably due to conformational changes or incorrect folding (*10*−*12*).

Binding of Monoclonal Antibodies. The binding of four function-blocking mAbs to the mutant CD59s was determined by flow cytometry. The results are summarized in Table 1.

All four mAbs bound well to the wild-type CD59, to the myc-tagged CD59, and to eight of the 21 single mutants. The black squares show that none of the mAbs bound to any of the D24, Y36, W40, or L54 mutants and that 1B4 and 3B4 also failed to bind either of the two K48 mutants. Mutations of I56 also inhibited binding of some of the mAbs with binding of 1B4, 3B4, and 6D1, but not TH9, reduced against the I56A mutant and the binding of all four mAbs markedly reduced against I56E mutations. Binding of 6D1 and 3B4 was reduced against the D44A mutant, binding of TH9 was lost, and only 1B4 retaining full binding. Conversely, only 1B4 binding was reduced by the S52A and E55A mutations.

Table 1: Binding of Anti-cmyc and Anti-Rat CD59 Antibodies to CHO Cells Expressing the Wild-Type and Mutated Rat CD59s, As Assessed by Flow Cytometry[a]

	c-myc	Poly	6D1	TH9	1B4	3B4
pDR2	7.5	10	8.0	3.5	3.5	4.6
Wild Type	5.8	469	678	377	582	1039
myc-wt	427	504	713	398	580	1209
S20A	378	485	653	372	528	1548
P21A	380	459	633	381	544	1502
N22A	391	508	641	378	554	1106
L23A	383	477	642	346	566	1055
D24A	509	487	7	18	5.8	23
D24R	436	536	13	9.5	10	35
Y36A	458	475	6.5	3	6.5	24
Q38A	401	507	696	389	601	1245
W40A	378	504	14	7	9.7	19
W40E	514	515	6	5	9	27
R41A	390	483	717	379	591	958
D44A	543	532	210	35	569	650
K48A	409	464	654	410	5	12
K48E	390	471	600	419	15	8
S52A	447	498	816	404	139	1176
S52D	434	506	796	390	568	1232
R53A	361	456	715	380	515	1057
L54A	422	442	4	2	3	5
E55A	382	466	685	389	163	976
I56A	523	479	217	395	125	493
I56E	411	521	102	47	42	194
K48E/I56E	460	506	89	40	6	10
S52D/I56E	488	534	208	25	53	536

[a] Mutants showing <10% reactivity compared to wild type with a given antibody are shaded black. Mutants showing 10−30% reactivity compared to wild type with a given antibody are shaded dark gray. Mutants showing 30−60% reactivity compared to wild type with a given antibody are shaded light gray.

Previously there has been no way to distinguish between these four blocking mAbs, but these data show conclusively that they each bind distinct but overlapping epitopes.

Function of Mutants. The complement regulatory function of all mutant rat CD59s was determined by measuring lysis of expressing cells by rat serum in a classical pathway assay. Expressing cells were also tested against human and rabbit serum in the same assay in order to determine if any of the mutations were in residues involved in the species selectivity of rat CD59.

Classical pathway activation of complement was initiated by presensitizing the cells with a rabbit polyclonal anti-CHO antiserum, and the cells were then exposed to a 1:10 dilution of serum for a length of time predetermined to give 70−80% lysis of the vector control cells (pDR2; Figure 1).

Under these conditions the wild-type and myc-tagged rat CD59 molecules both inhibited lysis by all three serum types to similar degrees (Figure 1), confirming that the incorporation of the myc tag into the molecule had no effect on function.

All of the mutations tested produced similar levels of inhibition with all three serum types, indicating that none of the residues was important in species selectivity. The majority of the mutations had little or no effect on the function of the molecule, inhibiting lysis to a similar degree as the wild-type CD59. Notable exceptions were the D24A,

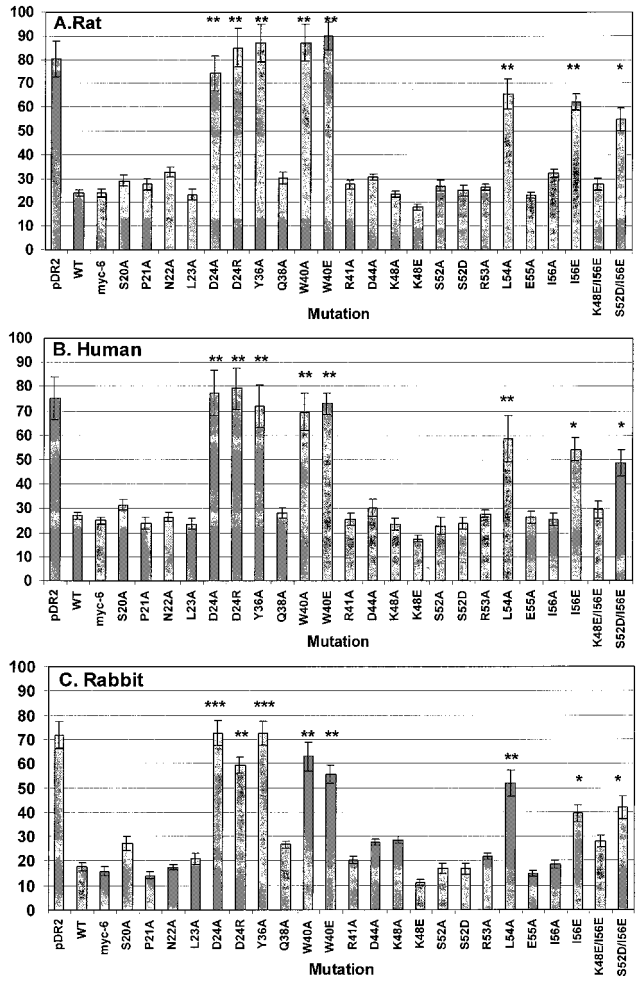

FIGURE 1: Protection of CHO cells expressing wild-type (wt), myc-tagged (myc-6), and mutant rat CD59s compared to the vector control transfected cells (pDR2). Cells were loaded with calcein-AM, presensitized with an anti-CHO polyclonal antiserum, and then incubated with a 1:10 dilution of (A) rat serum, (B) human serum, and (C) rabbit serum. Lysis by serum was then calculated as a percentage of total lysis by detergent. The bars represent the means and standard deviations of triplicate samples from a single experiment. Statistical analysis of significance of differences between groups in the experiment was performed with ANOVA with Bonferroni corrections. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Independent replications of the study, while differing in absolute levels of lysis, gave similar differences between groups.

D24R, Y36A, W40A, and W40E mutants, all of which failed to confer any complement resistance to the expressing cells. The L54A and I56E mutants and the S52D/I56E double mutant were partially functional, with reduced levels of lysis compared to the vector control cells, but were not as efficient as the wild-type CD59 at inhibiting all three serum types. The K48E single mutant had apparently increased function against all three serum types when compared to the wild-type CD59, although a K48A mutation had no effect on function. The K48E/I56E double mutant had similar inhibitory function to the wild-type CD59.

Increased Function of K48E. To confirm whether the K48E mutation had increased complement regulatory function, cells expressing wild-type (myc6) CD59 and those expressing K48A or K48E mutant CD59 were incubated with various dilutions of rat, human, and rabbit serum for an extended time. This allowed an increased lysis of expressing cells, thus augmenting any difference between the CD59

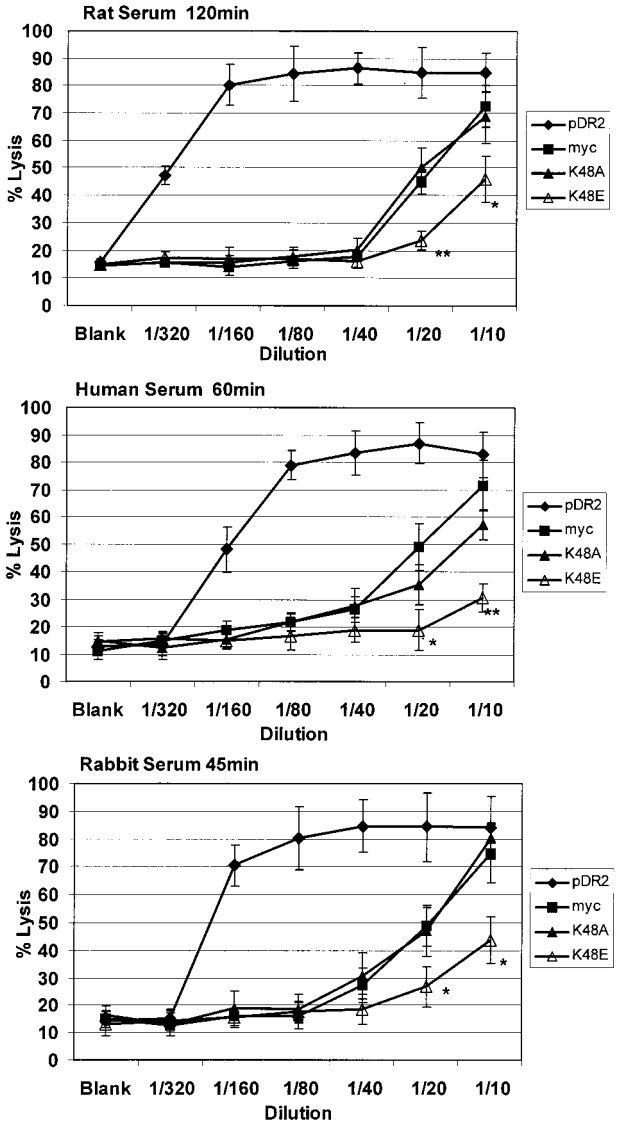

FIGURE 2: Comparison of the inhibitory function of the K48E and K48A mutants to the myc-tagged rat CD59. Expressing cells were loaded with calcein, antibody-sensitized, and then incubated with doubling dilutions of serum from (A) rat, (B) human, and (C) rabbit. The data points represent the means and standard deviations of triplicate samples from a single experiment. Statistical analysis of significance of differences between groups in the experiment was performed with ANOVA with Bonferroni corrections. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Independent replications of the study, while differing in absolute levels of lysis, gave similar differences between groups.

mutations. The results of this experiment are shown in Figure 2.

Cells expressing the K48E mutant CD59 consistently showed significantly lower levels of lysis by all three species sera when compared to wild-type CD59 or the K48A mutant. To achieve a given level of lysis of cells expressing the K48E mutant, approximately double the serum concentration was required as compared with cell expressing wild-type or K48A mutant CD59. This finding was true for all three species sera tested. It is clear from Table 1 that these data were not due to differences in relative levels of expression. In these experiments the K48A mutant showed a slight but nonsignificant increase in function against human serum compared to wild-type CD59, but no increase in function against rat and rabbit serum.

Table 2: Binding of Rabbit Polyclonal and Mouse Monoclonal Anti-Human CD59 Antibodies to the Wild-Type CD59 and the N48E Mutant As Assessed by Flow Cytometry

	Bric229	Mem43	YTH 53.1	poly
human	1291	785	472	824
N48E	1176	794	459	763

[a] Values shown are mean fluorescence units.

Human N48E Mutant. Substitution at residue 48 in rat CD59 of a basic residue with an acidic residue enhanced function. We wished to determine if a similar mutation at this site in human CD59 would increase function. In human CD59, residue 48 is N (polar/neutral). A N48E mutant was generated by two-stage PCR. Expression of this mutant in CHO cells with the pDR2ΔEF1α vector was compared to that of wild type by flow cytometry with a panel of mAbs. Both the wild type and N48E mutant were detected by all mAbs and expressed at similar levels (Table 2).

The function of the N48E mutant was compared to wild-type human CD59 in a calcein release assay, and the results are shown in Figure 3. The N48E mutation had no effect on the ability of human CD59 to inhibit human and rat serum. However, the N48E mutation completely abrogated the ability of human CD59 to inhibit lysis by rabbit serum.

DISCUSSION

The published data on the active site of human CD59 are confusing, with mutations of physically distant residues producing effects on the function of the molecule. Two groups proposed the hydrophobic groove centered on W40 as the active site (*11*, *12*). This proposal was, however, based upon relatively few mutations in this region. It was therefore decided to mutate this region more comprehensively, in a nonprimate species, to confirm conservation of this potential active site and to characterize it further. Rat CD59 was chosen in order to allow other mutations to be made to simultaneously address the identity of residues conferring species selectivity. Individual mutations were made in all residues in and around the groove and hydrophobic ridge, with the exception of the structurally important C39. Mutation of C39 in human CD59 prevented expression at the cell surface (*10*). All potential active-site residues were mutated to alanine, a small, neutral residue, in order to prevent major disruption to the protein that can occur with nonconservative mutations. In addition to these alanine substitutions, mutations of W40E and D24R were also generated to investigate whether these reproduced the loss of function for these mutations seen with human CD59 (*11*).

The face of the α-helix adjacent to the hydrophobic groove (human residues N48, T52, R55, and E56) is highly negatively charged in mouse (E48, D52, E55, and E56) and rabbit CD59 (E48, N52, E55, and E56), both of which are poor inhibitors of human complement but good inhibitors of rodent complement. This region is overall charge-neutral in both human and pig CD59 (D48, R52, A55, and E56), both of which are good inhibitors of human complement but poor inhibitors of rodent complement. Rat CD59 (K48, S52, E55, and I56) is a good inhibitor of both human and rodent complement and is also charge-neutral in this region. It is therefore possible that the net charge in this region is an important factor in species selectivity. Individual and double mutations from rat to mouse CD59 sequence were made in these residues of the α-helix in order to determine whether this would reduce the ability of rat CD59 to inhibit human complement.

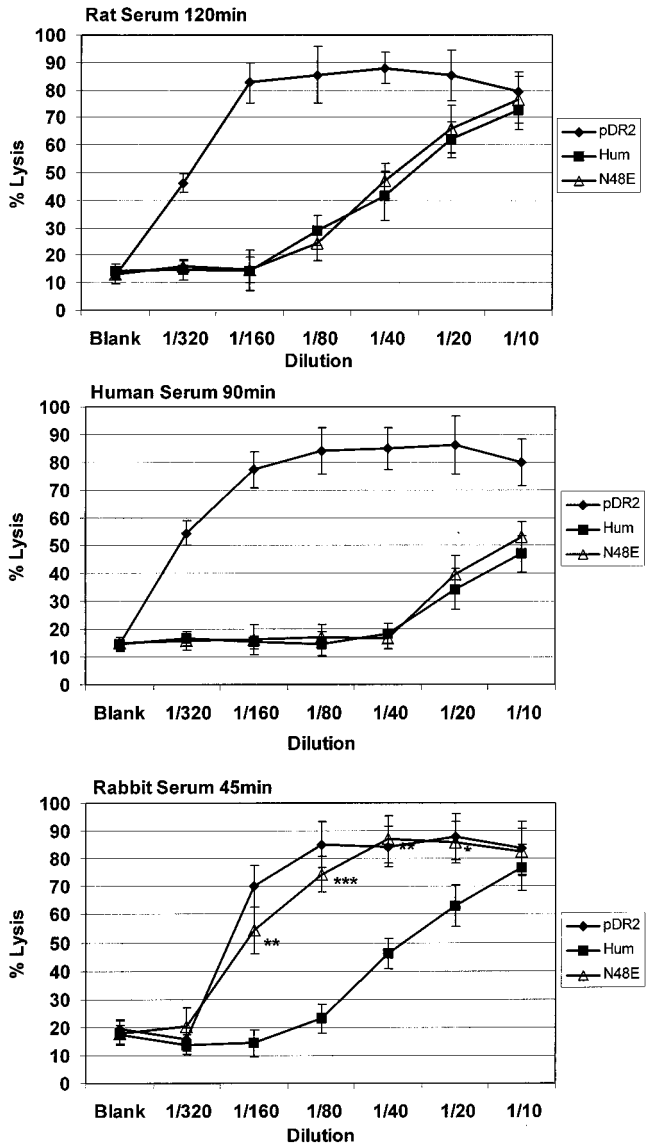

FIGURE 3: Comparison of the inhibitory function of the N48E mutant to the wild-type human CD59. Expressing cells were loaded with calcein, antibody-sensitized, and then incubated with doubling dilutions of serum from (A) rat, (B) human, and (C) rabbit. The data points represent the means and standard deviations of triplicate samples from a single experiment. Statistical analysis of significance of differences between groups in the experiment was performed with ANOVA with Bonferroni corrections. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$. Independent replications of the study, while differing in absolute levels of lysis, gave similar differences between groups.

All mutant CD59 molecules were abundantly expressed on the CHO cell surface and were correctly folded, as shown by the binding of both anti-c-myc and polyclonal anti-rat CD59 mAbs. This in itself was a surprising result. Some of the mutations generated were thought likely to cause disruption to the structure of the active site, and thus the conformation of the whole molecule might be affected. It was therefore expected that some of the mutations would not fold correctly and would not be expressed on the cell

surface. This has previously been shown for several mutations in human CD59, particularly those in W40 (*10*−*12*).

The binding of all available anti-rat CD59 mAbs to the mutant CD59 expressing CHO cells was assessed by flow cytometry. All four of the mAbs were function-blocking antibodies, indicating that their epitopes were close to the active site enabling antibody binding to sterically block subsequent binding of C8 and/or C9 to the CD59 molecule. No non-function-blocking mAbs against rat CD59 were available for comparison. The data produced by the binding of mAbs to the mutant CD59s locates the epitopes of these mAbs to a region centered on the hydrophobic groove containing W40 and the adjacent α-helix, reproducing the results obtained with human CD59 and further implicating this region as the active site. The data showed that all four mAbs have very close, but distinct epitopes. None of the four mAbs bound to cells expressing CD59 with mutation in the residues of the hydrophobic groove (Y36, W40, and L54). Some of the mutations of the adjacent α-helix also disrupted the binding of some of the mAbs. Two of the four mAbs (1B4 and 3B4) failed to bind either of the K48 mutants, and binding of all four was low on the I56 mutants. The binding of 1B4 was reduced by the α-helix mutations S52A and E55A but not by S52D. Binding of the other mAbs was unaffected by mutations in S52 and E55. Mutations in the hydrophilic ridge adjacent to W40 (R41, D44, R53, and E55) had little effect on the binding of the mAbs, except for the D44A mutation, which reduced the binding of all mAbs except 1B4. Mutations of the 20−24 loop also had no effect on mAb binding, except for the D24A and D24R mutations. These mutations prevented binding of any of the four mAbs. This result is difficult to explain as this residue is some distance from the other residues that influence mAb binding, but is consistent with the finding for human CD59, where a D24R mutation influenced the binding of mAbs whose epitopes were centered around the hydrophobic groove (*11*).

Mutations in rat CD59 that adversely affected the binding of the four mAbs also inhibited the function of the molecule. All the hydrophobic groove mutations (Y36A, W40A, W40E, and L54A) ablated function, suggesting that these residues play a direct role in binding and confirming the human CD59 data (*10*−*12*). We also addressed the possibility that the 20−24 loop was involved in function. The D24A and D24R mutations both completely abolished function, as was seen with human CD59, but alanine substitutions in the other four residues in this loop had no effect on function. In human CD59, F23R and F23D mutations also reduced function of the molecule (*11*, *12*). This discrepancy may be due to the nonconservative substitutions in human CD59 disrupting adjacent regions of the molecule. The influence of the D24 mutations on binding of the four mAbs, the epitopes for which are on the other side of the molecule, and effect on function of the molecule suggests that the D24 residue is crucial for the structure and/or accessibility of the active site. Of note, the D24 mutations had no effect on the binding of the polyclonal anti-rat CD59; thus the conformational change cannot affect the whole molecule.

Somewhat surprisingly, none of the mutations of the hydrophilic ridge, adjacent to the hydrophobic groove, had any effect on the function of the molecule. The hydrophilicity of this ridge is highly conserved across species, and we thus anticipated an involvement in function. It remains possible that the ridge is involved in C8/C9 binding but mutation of a single residue in this region is not enough to prevent binding, provided that the overall hydrophilicity of the ridge is conserved.

The α-helix (residues 42−58) has been implicated in both function and species selectivity of human CD59. The adjacent residues F47 (α-helix) and Y61 (triple *β*-sheet) were implicated as key to activity in separate studies (*10*, *14*). F47 was shown to be important in conferring species selectivity (*14*). In the current study, neutral mutations in the α-helix were made to assess both influence on activity (Ala substitutions) and role in species selectivity (single and double mutations to mouse sequence). Of the Ala substitutions, only L54A had a significant negative effect on function, similar for all sera tested. Of the mutations to mouse sequence, none of the single or double mutations conferred mouselike activity (inhibiting rodent but not human serum) on rat CD59. However, I56E (aliphatic to acidic) markedly reduced the inhibitory properties of rat CD59 for all sera tested, although some function was retained for all sera. Bodian et al. (*11*) previously showed that the E56R (acidic to basic) mutation in human CD59 completely abrogated function. The loss of function caused by nonconservative mutations of residue 56 suggests either that the active site of CD59 extends into the α-helix or that mutations in this region cause conformational changes in the hydrophobic groove.

Further evidence implicating the α-helix in the function of CD59 is provided by the observation that rat CD59 K48E mutation caused an increase in function of the molecule compared to the wild-type CD59 against all three serum types tested (Figure 2). A similar mutation of residue 48 in human CD59 (N48E) had no effect on the function of the molecule against rat or human serum but dramatically reduced its efficiency at inhibiting rabbit serum (Figure 3). This was unexpected, as the rabbit CD59 sequence codes for E at residue 48 and thus this mutation would be predicted to make human CD59 more like rabbit CD59, which can efficiently inhibit rabbit serum (*5*). The propensity of mutations at residue 48 to influence activity and species selectivity may be due to its position in the α-helix. Located at the beginning of the helix, mutations may cause a conformational change, thus altering the positions of side chains all along the helix. Side chains projecting from the helix may interact with C8/C9 and either permit or bar stable binding in the hydrophobic groove. From these data it can be concluded that the amino acid residues that determine species selectivity are likely to differ between species, and within a particular CD59 molecule they may differ depending on the source of complement.

In conclusion, we have identified residues in rat CD59 that are important in its complement regulatory activity (active site). We have also identified a single residue of human CD59 (N48) that influences the interaction with rabbit C8/C9. We found that a K48E mutation of rat CD59 caused an increase in function of this molecule and highlighted the key role of the α-helix between residues 46 and 62. Further mutagenesis in this region may lead to the identification of yet more potent forms of CD59. Engineering more potent forms of CD59 may guide the development of MAC inhibitors for use in therapy of complement-mediated diseases.

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