Mapping the Regions of the Complement Inhibitor CD59 Responsible for Its Species Selective Activity†

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ABSTRACT: CD59 is a widely distributed membrane-bound glycoprotein that inhibits the formation of the cytolytic membrane attack complex (MAC) of complement on host cells. CD59 from different species varies in its capacity to inhibit heterologous complement, and this species selective function of CD59 contributes to the phenomenon of homologous restriction. Here, we demonstrate that human CD59 is not an effective inhibitor of rat complement, although rat CD59 inhibits rat and human complement equally well. By constructing human−rat CD59 chimeric proteins, we have mapped the residues important in conferring human CD59 species selectivity to two regions; 40−47 and 47−66 in the primary structure. Analysis of a model of the molecular surface of human CD59 revealed that residues 40−66 mapped to a region in the three-dimensional structure that surrounds residues previously identified as important for CD59 function.

Activation of complement results in the formation of C3/C5 convertase enzymes on the activating surface. The convertases serve to amplify the cascade and may lead to the assembly of the terminal complement proteins which form the cytolytic membrane attack complex (MAC).[1] Host cell membranes are protected from the proinflammatory and potentially cytolytic effects of the MAC by CD59, a widely distributed glycosylphosphatidylinositol (GPI)-linked membrane glycoprotein. CD59 functions by binding the terminal complement proteins C8 and C9 in the assembling MAC and interfering with its membrane insertion (*1*−*4*).

An important feature of CD59 and other complement inhibitors is their species selectivity. This feature is responsible for the phenomenon of homologous restriction, whereby cells are largely resistant to lysis by homologous complement. The selective activity of CD59 is due to the species selective recognition of C8 and/or C9 in the assembling MAC. The species selective activity of CD59 appears to be well illustrated by the prolonged survival of transgenic pig organs expressing human CD59 following their transplantation into baboons (*5*, *6*). Under normal circumstances, complement is involved in the rapid destruction of xenotransplanted tissue, a phenomenon known as hyperacute rejection. Nevertheless, species selective recognition is not absolute, and CD59's from different species vary in their effectiveness at inhibiting heterologous complement (*1*, *7*−*12*).

Mutational analysis of human CD59 has recently identified residues important for human CD59 function (*13*, *14*). In the current study, we begin to define the structural basis for the species selective activity of human CD59. Utilizing a quantitative difference in the species selective activity of human and rat CD59, we have mapped functionally important residues to sites that lie in the vicinity of the broadly defined region of the CD59 active site.

EXPERIMENTAL PROCEDURES

Cells and DNA. Human and rat cDNA subcloned into the mammalian expression vector pCDNA3 (Invitrogen, Carlsbad, CA) was used for all DNA manipulations. Human CD59 cDNA was a gift from H. Okada (Nagoya City University, Nagoya, Japan), and rat CD59 cDNA was isolated as described previously (*15*). Chinese hamster ovary cells (CHO) were used for protein expression and were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% FCS. pCDNA3 contains a G418 resistance marker, and stably transfected CHO cell clones and populations were selected following the cultivation of cells in the presence of G418 (Gibco, Gaithersburg, MD).

Antibodies and Sera. Rabbit antiserum to CHO cell membranes that was used to sensitize CHO cells to complement was prepared by standard techniques (*16*). CHO cell membranes were prepared as described (*17*). Anti-rat CD59 monoclonal antibody 6D1 was described previously (*18*). mAb 2A10 (*19*) is directed against $(NANP)_n$, a repeat domain of *Plasmodium falciparum* circumsporozoite protein, and was used to quantitate expression of epitope-tagged proteins. FITC-conjugated Abs used for flow cytometry were purchased from Sigma (St. Louis, MO). Normal human serum

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[1] Abbreviations: MAC, membrane attack complex; FCS, fetal calf serum; CHO, Chinese hamster ovary; mAb, monoclonal antibody; DMEM, Dulbecco's modified essential medium.

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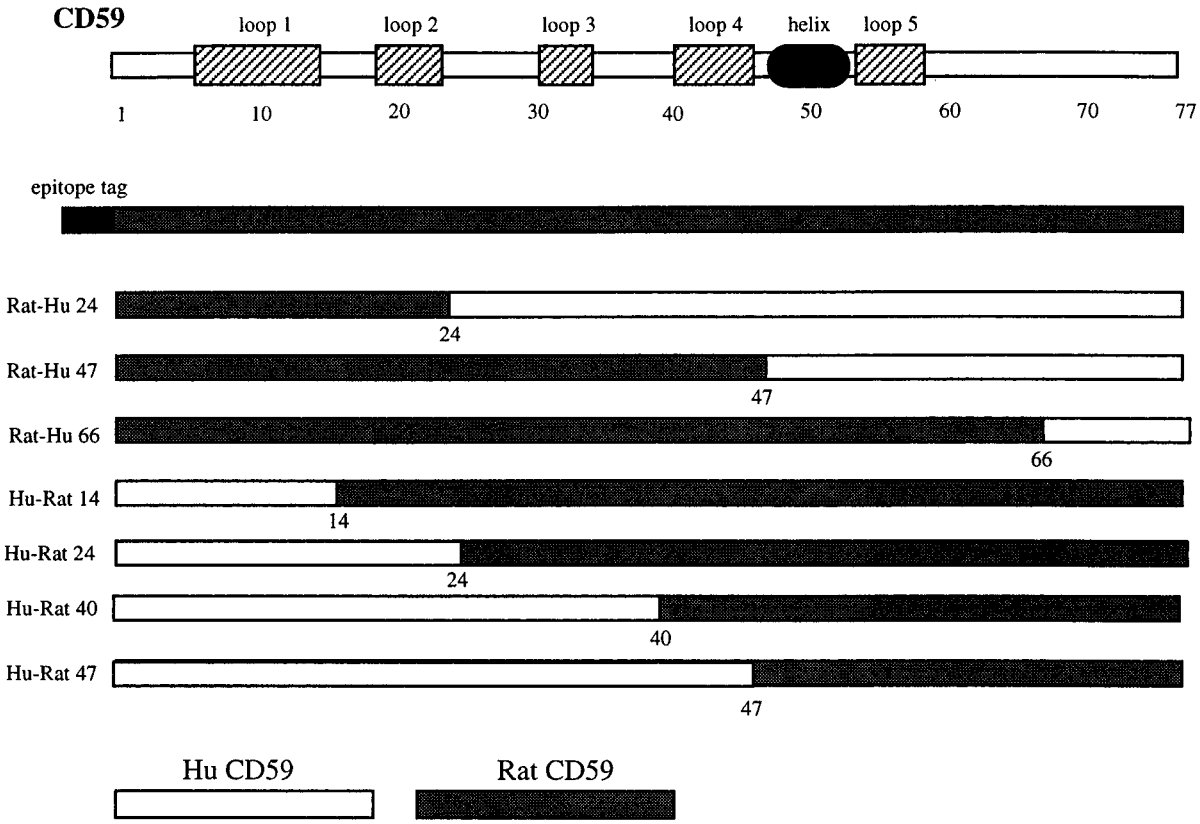

FIGURE 1: Mutant diagram. Diagram of human−rat chimeric constructs that were prepared and expressed on CHO cells. Numbers refer to amino acid positions which correspond in both human and rat sequences.

(NHS) was obtained from the blood of healthy volunteers in the laboratory. Freshly collected rat serum was purchased from Cocalico Biologicals (Reamstown, PA). All sera were stored in aliquots at −70 °C until they were used.

Construction of Chimeric Proteins. cDNA encoding the chimeric human−rat CD59 protein constructs shown in Figure 1 was prepared using PCR amplification by procedures previously described (*14*, *20*). Fragments from either human or rat CD59 cDNA that were to be fused were generated separately through amplification of cDNA cloned into the mammalian expression vector pCDNA3. The 5′ and 3′ end primers, which matched an untranslated region of either human or rat cDNA, and which included a *Hin*dIII and *Apa*I site, respectively, were paired with a "bridge" primer that spanned the junction. With primers corresponding to 5′ or 3′ untranslated regions, the PCR products were used in a second PCR to generate the chimeric cDNA. The resultant chimeric cDNA was cloned into the *Hin*dIII/*Apa*I sites of pCDNA3 for sequencing and expression. To quantitate the relative expression of recombinant proteins, an epitope tag consisting of amino acids NANPNANPNA was inserted at the N terminus of all recombinant proteins as described for human CD59 (*14*).

Preparation and Analysis of Cells Expressing Recombinant Proteins. pCDNA3 constructs were transfected into 50−75% confluent CHO cells using lipofectamine according to the manufacturer's instructions (Gibco). Stable populations of CHO cells expressing similar levels of tagged recombinant protein were isolated by several rounds of cell sorting by means of anti-tag mAb 2A10 as described (*14*). Three to four rounds of sorting were necessary to obtain homogeneous cell populations. Analysis of cell surface protein expression was performed by flow cytometry using anti-tag mAb 2A10 and anti-rat CD59 mAb 6D1. Cell sorting and flow cytometry were performed as previously described (*14*). Stably transfected CHO cell clones expressing rat CD59 and N-terminus-tagged rat CD59 were prepared according to instructions supplied by Gibco with lipofectamine reagent.

Cell Lysis Assay. Subconfluent CHO cells were detached with versene (Gibco), washed once, and resuspended to 1 $\times$ 10^6/mL in DMEM/10% heat-inactivated FCS. Rabbit anti-CHO cell membrane antiserum was added to 20%, and the cells were incubated on ice for 30 min. The cells were centrifuged and resuspended to their original concentration in DMEM/10% FCS. Equal volumes of cells and serum dilutions were incubated for 60 min at 37 °C, and cell lysis was then determined by one of two methods. In the first method, cells were washed once with DMEM and resuspended to 1 $\times$ 10^6/mL in DMEM and an equal volume of calcein-AM was added (5 μg/mL) (Molecular Probes, Eugene, OR). After 15 min at room temperature, viable cells were assessed by measuring fluorescence in microtiter plates using a Labsystems fluoroscan II instrument set to read excitation at 485 nm and emission at 538 nm. Cells were lysed with 0.01% saponin for 100% lysis controls. Calcein-AM is taken up by viable cells where it it is cleaved by intracellular esterases to yield a fluorescent derivative. This procedure measures fluorescence within viable cells. Cell lysis was also determined by measuring the uptake of propidium iodide by dead and damaged cells using flow cytometry (*14*). Both assay methods gave similar results.

Molecular Modeling. A CD59 analytical molecular surface, which is defined as the smooth envelope touching the van der Waals surface of atoms as the solvent probe rolls over the protein molecule, was built as previously described (*14*).

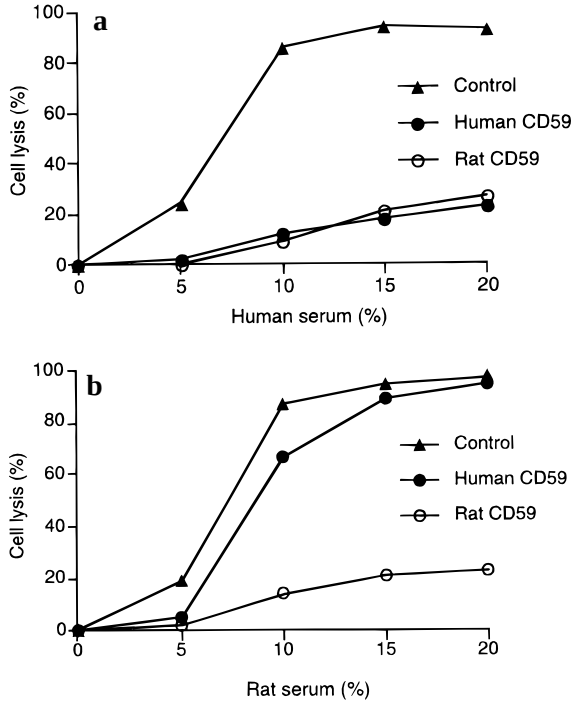

FIGURE 2: Activity of human and rat CD59 against homologous and heterologous complement. Stable CHO cell populations expressing similar levels of either human or rat CD59 were exposed to the indicated concentrations of either human (a) or rat (b) serum and percent lysis determined. Representative of five separate experiments.

Table 1: Flow Cytometric Analysis of Stably Transfected CHO Cells

	relative mean fluorescence	
expressed protein	anti-tag mAb (2A10)[a]	anti-rat CD59 mAb (6D1)
control CHO cells	15	11
stable clones		
untagged rat CD59	–	697
tagged rat CD59	1108	753
stable populations		
human CD59	1170	–
rat CD59	1229	–
rat−human 24	1272	–
rat−human 47	1149	–
rat−human 66	1105	–
human−rat 14	1096	–
human−rat 24	1304	–
human−rat 40	1300	–
human−rat 47	1289	–

RESULTS

Species Selective Activity of Human and Rat CD59. Stably transfected CHO cells expressing rat CD59 were effectively protected from both rat and human complement (Figure 2). On the other hand, human CD59 expressed on CHO cells was effective at inhibiting human complement, but much less effective at inhibiting heterologous rat complement (offering no protection from lysis by 20% rat serum). Rat and human CD59 were equally effective at inhibiting human complement, since CHO cell populations expressing similar levels of each protein displayed similar levels of resistance to human serum (Table 1 and Figure 2).

To quantitate the relative surface expression of recombinant human and rat CD59 and the chimeric proteins described below, an amino acid epitope tag (NANPNANPNA) was engineered onto the N termini of all constructs. We have shown previously that this tag does not affect the activity of human CD59 expressed on the surface of CHO cells (*14*), and Figure 3 shows that the tag similarly has no effect on rat CD59 activity.

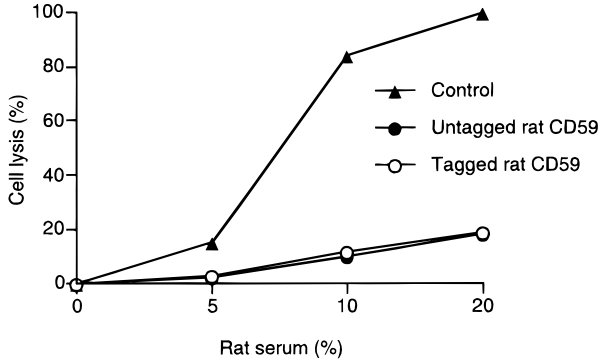

FIGURE 3: Relative activity of native and epitope-tagged rat CD59 expressed on the surface of CHO cells. Stable CHO cell clones expressing similar levels of rat CD59 or rat CD59 containing an N-terminal NANPNANPNA peptide tag (see Table 1) were assayed for their susceptibility to complement. Representative of three separate experiments.

Complement Inhibitory Activity of Human−Rat CD59 Chimeric Proteins. We made use of the species selective nature of human CD59 activity to define functionally important regions of the molecule. The epitope-tagged chimeric human−rat CD59 constructs depicted in Figure 1 were prepared, with human−rat switchover points chosen to lie at boundaries of surface-exposed loops (*21*, *22*). The constructs were stably transfected into CHO cells, and cell populations expressing similar levels of recombinant proteins were isolated (Table 1) and assayed for susceptibility to both human and rat serum.

All of the chimeric proteins expressed on CHO cells were as active against human complement as native human and rat CD59, indicating that the proteins were all correctly folded (Figure 4a). Of the chimeric proteins containing rat-specific amino acids at their N termini, only rat−human 66 (N-terminal 66 residues of rat sequence) was fully active against rat complement. Rat−human 24 had activity comparable to that of human CD59 and had little or no activity against 20% rat complement (Figure 4b). These data indicate that the residues that are important for conferring species selective activity on human CD59 are located between positions 24 and 66 in the primary structure. The full or very near full functional activity of human−rat 14, 24, and 40 (N-terminal human-specific residues) against rat complement indicates that only the C-terminal half of human CD59 (40−77) is involved in its species selective activity. These data indicate that the species selective functional region of CD59 is located between residues 40 and 66 in the primary structure.

Both rat−human 47 and the converse construct human−rat 47 possessed an intermediate level of activity against rat complement (Figure 4b). Considered together with the above data, this indicates that residues in more than one region are responsible for conferring species selective function on human CD59; within the sequence 40−66 identified above, one region maps to residues 40−47 and one to 47−66 (see Figure 5).

Location of Residues That Are Important for Species Selective Activity. On the basis of the known structure of

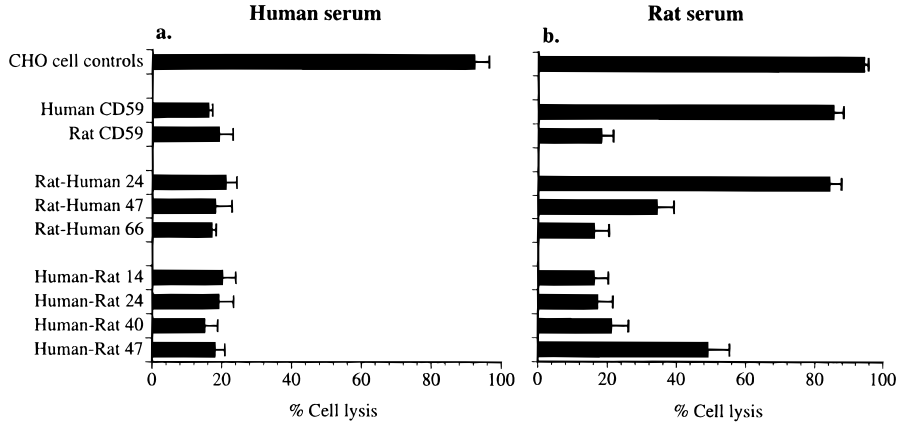

FIGURE 4: Complement resistance of CHO cells expressing human and rat CD59 and human−rat chimeric CD59 proteins. Stable CHO cell populations expressing similar levels of recombinant protein were exposed to 20% human serum (a) or rat serum (b) and percent lysis determined. Mean ± SD. $n = 5$.

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          1          10          20          30
Human  LQCYNCPNPTADCKTAVNCSSDFDACLITKAGLQVYNKC
       :  ::::   :    :::    ::    ::::     : :::  :
Rat    LRCYNCLDPVSSCKTNSTCSPNLDACLVAVSGKQVYQQC

          40          50          60          70
Human  WKFEHCNFNDVTTRLRENELTYYCCKKDLCNFN-EQLEN
       :  :   ::      ::       :  ::   ::::   :   :
Rat    WRFSDCNAKFILSRLEIANVQYRCCQADLCNKSFEDKPN
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FIGURE 5: Sequence alignment of human and rat CD59. Residues within the two regions determined to be important in conferring species selective function are shown in bold. Residues of one region (40−47) are underlined. Mature protein sequences are shown, and identical residues are marked (:). The C-terminal end of rat CD59 is predicted.

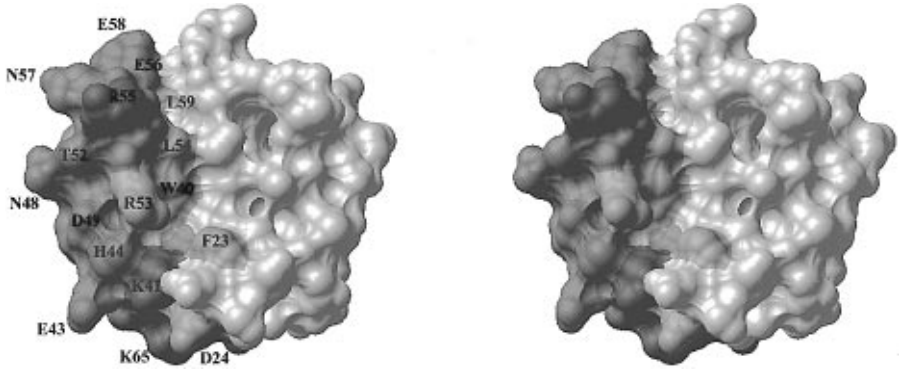

FIGURE 6: Stereodiagram showing the molecular surface of human CD59 and functionally important residues. The surface of regions 1−39 and 66−77 that are not important for species selectivity is colored white. Residues that are conserved with rat CD59 and that were also previously identified as functionally important in human CD59 are colored magenta. Side chains of all residues potentially responsible for species selective activity (fragment 40−66) are colored red (negatively charged residues), blue (positively charged residues), yellow (hydrophobic residues), and green (other residues). Backbone atoms of other residues, as well as side chains of residues that are identical in human and rat CD59 and not important for species selectivity, are colored gray.

human CD59 (*21*, *22*), a model of the molecular surface of human CD59 was built and the three-dimensional location of the identified regions that are important for species selectivity determined. First, we analyzed the three-dimensional regions of CD59 that do not appear to be involved in species selective activity, i.e. residues 1−39 and 66−77 (see Figure 5). These regions are shown in white in Figure 6 and map to the back and front right side of the shown face. The face of the molecule shown is predicted to face away from the membrane and contains the residues that previous studies indicate are important for human CD59 function (*13*, *14*). These previously identified functionally important human residues are E56 (not conserved with rat) and F23, D24, W40, R53, and L54 (conserved, or map to region not involved in species selective activity, shown in magenta in Figure 6), and they map to the vicinity of an almost linear groove in the exposed face of the molecule (Figure 6). Next, the surface shape and the distribution of surface patches of human CD59 within the region containing the residues that are important for species selective activity were analyzed. These residues (40−66, see Figure 5) are shown in color in Figure 6, and they map to one side of the molecule, partially surrounding the functionally important human CD59 residues in the vicinity of the above identified

groove. The residues shown in gray are the same in human and rat CD59 (see the legend to Figure 6 for an explanation of the colors).

DISCUSSION

The effectiveness of CD59 from human and other species at inhibiting heterologous complement varies widely and has been the subject of some controversy. In this report, we demonstrate on a quantitative basis the species selective activity of human CD59 for human versus rat complement and show that human and rat CD59 are equally effective against human complement. We next addressed the identification of the site(s) involved in the species selective activity of human CD59. By measuring the activity of human−rat CD59 chimeric proteins, we have mapped the responsible residues to positions 40−66 in the primary structure. Significantly, this region corresponds to a region of particularly low sequence homology (34% compared to 51% for rest of the sequence). Our data further indicate that there are at least two sites within the 40−66 region that confer species selectivity: one site between residues 40 and 47 and another between 47 and 66 (refer to Figure 5).

We have separately reported elsewhere that the mutation of certain residues contained within the human CD59 sequence of 23−56 results in a loss of activity (*13*, *14*), and that the 20 C-terminal amino acids and the 16 N-terminal amino acids of human CD59 do not appear to be required for function (*14*). These previous data map the active site residues of CD59 to positions in and around a conserved hydrophobic groove on the exposed, membrane distal face of the molecule (see Figure 6). Experimental data reported here indicate that separate residues or residue groups within the two regions of 40−47 and 47−66 are responsible for conferring species selective function, and these residues map to a region directly above, below, and extending along one side of the groove (see Figure 6).

Comparative analysis of the rat and human CD59 sequence within the 40−66 region revealed pronounced differences in the residues that map to regions above and below the identified groove (refer to Figures 5 and 6). In the upper region, the six human residues N48, T52, R55, E56, N57, and E58 (Figure 6) will produce surface patterns of electrostatic potential and hydrophobicity very different from those of the corresponding rat residues, K48, S52, E55, I56, A57, and N58 (refer to Figure 5). Position 55 is the most protruding and is likely to be in contact with the C8/C9 ligand. In the lower region, side chains at positions 41, 43, 44, and 49 form another cluster of differences in shape and electrostatic properties in the vicinity of the groove. These residues are K, E, H, and D and R, S, D, and F for human and rat CD59, respectively. This lower site lies within the experimentally determined important 40−47 region and thus very likely contributes to species selectivity. Finally, at the very bottom of the groove, a positively charged residue K65 in human is replaced by a polar Q65 in rat CD59. This residue is placed next to the conserved and functionally important residue D24 (*13*).

In support of our current and previous data indicating the importance of residues within the 16−66 region for overall functional activity (*13*, *14*), Petranka et al. (*23*) have reported that disulfides Cys6−Cys13 and Cys64−Cys69 are not essential for CD59 function. These disulfides appear to stabilize structures distant from the hydrophobic groove identified above (*21*, *22*). However, additional mutational analyses by Petranka et al. (*23*) are at present difficult to reconcile with some of our previous data (*13*, *14*), and their data indicate that residue Tyr61 (on the membrane proximal face of the molecule) is important for function.

These results represent a first approximation at defining the species selective functional site of CD59 and lay a rational foundation for future mutational studies aimed at precisely defining the functionally important residues of CD59. Further study will determine whether separate species selective recognition and active sites exist on CD59, and the current data do not preclude this possibility. If there are indeed distinct recognition and active sites, it may be possible to design either a nonselective or a species selective efficient inhibitor of complement. It may also be possible to enhance the complement inhibitory activity of CD59 by substituting residues involved only in species selective recognition. CD59 binds both C8 (C8α chain) and C9 (*1*−*4*), and it is not known whether C8 and C9 share the same binding site on CD59. Nevertheless, whether or not separate sites exist, earlier functional studies suggest that human CD59 interacts with both C8 and C9 in a species selective manner (*1*).

A detailed knowledge of how CD59 functions may allow the design of a more efficient complement inhibitor, as well as the design of inhibitors of CD59. Recombinant complement inhibitors have been shown to effectively reduce the pathological consequences of inflammation in various animal models of disease and show promise in the treatment of autoimmune and inflammatory disease (*24*−*28*). Another potentially important use for complement inhibitors is prevention of hyperacute rejection of xenotransplants (*6*, *29*−*31*). Hyperacute xenograft rejection is due to MAC-mediated tissue destruction following complement activation by xenoreactive natural antibodies (*32*). On the other hand, inhibitors of complement inhibitory molecules may have uses in the treatment of cancer. Inhibiting CD59 or other complement inhibitors on tumor cells may enhance the therapeutic potential of antitumor antibodies.

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