Formation of the cytolytic membrane attack complex of complement on host cells is inhibited by the membrane-bound glycoprotein, CD59. The inhibitory activity of CD59 is species restricted, and human CD59 is not effective against rat complement. Previous functional analysis of chimeric human/rat CD59 proteins indicated that the residues responsible for the species selective function of human CD59 map to a region contained between positions 40 and 66 in the primary structure. By comparative analysis of rat and human CD59 models and by mutational analysis of candidate residues, we now identify the individual residues within the 40–66 region that confer species selective function on human CD59. All nonconserved residues within the 40–66 sequence were substituted from human to rat residues in a series of chimeric human/rat CD59 mutant proteins. Functional analysis revealed that the individual human to rat residue substitutions F47A, T51L, R55E, and K65Q each produced a mutant human CD59 protein with enhanced rat complement inhibitory activity with the single F47A substitution having the most significant effect. Interestingly, the side chains of the residues at positions 47, 51, and 55 are all located on the short single helix (residues 47–55) of CD59 and form an exposed continuous strip parallel to the helix axis. A single human CD59 mutant protein containing rat residue substitutions at all three helix residues produced a protein with species selective activity comparable to that of rat CD59. We further found that synthetic peptides spanning the human CD59 helix sequence were able to inhibit the binding of human CD59 to human C8, but had little effect on the binding of rat CD59 to rat C8.

Complement activation can lead to the formation of the proinflammatory and cytolytic complement membrane attack complex (MAC) (4) (or C5b-9) on cell membranes, and inappropriate MAC formation on host cell membranes has been implicated in the pathogenesis of various autoimmune and inflammatory diseases. Host cells are normally protected from the effects of the MAC by CD59, a widely distributed membrane-bound glycoprotein.

The mature CD59 protein consists of 77 amino acids arranged in a single compact cysteine-rich domain composed of two antiparallel β-sheets, five protruding surface loops, and a short helix (1, 2). CD59 functions by binding the terminal complement proteins C8 and C9 in the assembling MAC and interfering with its membrane insertion (3–6). Because of species selective recognition of C8 and/or C9 (3, 7), the activity of CD59 is species restricted. However, species restriction is not absolute, and the effectiveness of CD59 from different species against heterologous complement varies.

Mutational analysis of CD59 has begun to define residues important for its complement inhibitory function. Two basic strategies have been used. In one approach, mutagenesis of human CD59 was used to determine protein regions and amino acids essential for its inhibitory function against human complement (8–10). These studies have putatively mapped the human CD59 active site to one side of the protein that contains the short helix. Most of the identified functionally important human residues are well conserved between species and are located in the vicinity of a hydrophobic cleft on the membrane-distal face of the protein (8). In a second related approach, residues important for species selective function have been identified by functional analysis of chimeric human/animal CD59 proteins (11, 12). It is not clear whether CD59 from different species share a common ligand binding site with species selective binding determined by other residues via indirect or allosteric mechanisms, or whether the residues involved in CD59 species selectivity are directly involved in ligand binding.

In a quantitative study on the species selectivity of human and rat CD59, it has been shown that human CD59 is not effective against rat complement, but that rat CD59 is equally effective against rat and human complement (11). Functional analysis of human/rat CD59 chimeric proteins has indicated that the residues responsible for the species selective activity of human CD59 lie between positions 40 and 66 in the primary structure (11). Consistent with this conclusion, a more recent study using chimeric human/rabbit CD59 indicated that sequence between residues 42 and 58 determine human CD59 species selectivity (12). In the current study, we identify individual residues involved in the species selective function of human CD59.

EXPERIMENTAL PROCEDURES

Materials—Human CD59 cDNA was a gift from H. Okada (Nagoya City University, Nagoya, Japan) and the isolation of rat CD59 was described previously (13). The mammalian expression vector pCDNA3 containing the G418 selection marker (Invitrogen, Carlsbad, CA) was used for all DNA manipulation and recombinant protein expression. All DNA primers used in PCR-based mutagenesis procedures were synthesized by Integrated DNA Technologies. Inc. (Coralville, IA). Recomb...
nant CD59 proteins were expressed in Chinese hamster ovary cells (CHO) that were maintained in Dulbecco’s modified essential medium containing 10% heat-inactivated fetal calf serum. Rabbit antiserum to CHO cell membranes (14) was prepared as described (15). Anti-tag monoclonal antibody 2A10 directed against NAP(NP)NA, a repeat domain in Plasmodium falciparum circumsporozoite protein, was purchased previously (4). Fluorescein isothiocyanate-conjugated antibodies used for flow cytometry were from Sigma. Rat C8 was purified as described (16). Recombinant soluble rat and human CD59 was expressed in CHO cells and purified by affinity chromatography as described (2). Human C8 was purchased from Advanced Research Technologies (San Diego, CA). Four CHO sequence specific peptides were synthesized and high pressure liquid chromatography-purified (>80%) by Genemed (South San Francisco, CA); peptide 1, RLEAELTY; peptide 2, FNDVTLRNELTY; peptide 3, WKEFHCNFNDVTRNLRELT and peptide 4, FNDVTLRI. Normal human serum was obtained from the blood of healthy volunteers in the laboratory. Rat serum was purchased from Cocalico Biologicals (Reamstown, PA).

Construction of Mutant CD59 Proteins—Residue substitutions in human CD59 were prepared by standard PCR mutagenesis techniques as described (8, 11). In the first PCR amplification, 5’ and 3’ primers matching an untranslated region of human CD59 and containing a HindIII and Apal site, respectively, were paired with primers spanning the target site in which a rat amino acid codon was substituted. Each final PCR product was digested with HindIII and Apal and then cloned into pCDNA3 expression vector for sequencing and expression. To quantify the relative expression of recombinant proteins, an oligonucleotide encoding the tag-peptide sequence NAP(NP)NA was inserted into the human CD59 N-terminal Leu codon as described (8).

Expression of Recombinant Proteins—CHO cells were transfected with pCDNA3 constructs using LipofectAMINE™ according to the manufacturer’s instructions (Life Technologies, Inc.). Stable transfectants were selected by the addition of G418 (400 μg/ml) 3 days after transfection. After 14 days of selection, stable populations of CHO cells each expressing similar levels of tagged recombinant protein were sorted by flow cytometry by means of anti-tag monoclonal antibody 2A10 as described (8). At least three rounds of cell sorting were required to obtain homogeneous cell populations expressing similar levels of recombinant protein.

Flow Cytometry—For quantitative analysis of tagged recombinant protein expression, stably transfected detached CHO cells were incubated with monoclonal antibody 2A10 (10 μg/ml) for 30 min at 4 °C. Cells were then washed, and incubated with fluorescein-conjugated anti-mouse IgG for 30 min at 4 °C. Cells were then washed again, fixed with 2% paraformaldehyde in phosphate-buffered saline and analyzed using a Becton Dickinson FACScan. All incubations and washing were performed as described previously (8). Briefly, cells were incubated in 20% heat-treated anti-CHO antiserum, washed once, and exposed to 20% human or rat serum (either active or heat-inactivated). Cell lysis for sorting were fluorescently labeled as above but were not fixed. Sorting was done in a Coulter Epics Elite with EPS sort module (Coulter Corp., Miami, FL).

Cell Lysis Assay—Complement-mediated CHO cell lysis assays were performed as previously described (8). Briefly, cells were incubated in 20% heat-treated anti-CHO antiserum, washed once, and exposed to 20% human or rat serum (either active or heat-inactivated). Cell lysis was determined by both trypan blue exclusion and by measuring the release of a preloaded fluorescent probe, calcein-AM (8). Both methods gave similar results. Lysis was determined using sets of homogeneous cell populations expressing similar levels of rat CD59, human CD59, or chimeric CD59 on their surface (Ref. 11 and also see above).

CD59 Binding Assay—The ability of synthetic human CD59 peptides to inhibit the binding of CD59 to its ligand C8 was determined using a previously described microtiter plate binding assay (4). Briefly, human or rat C8 was coated onto microtiter wells, and the respective binding of biotinylated human or rat CD59 was determined in the presence of varying concentrations of peptide. CD59 (at final concentration of 20 μg/ml in phosphate-buffered saline containing 0.1% bovine serum albumin) and different concentrations of peptide were mixed before addition to C8 coated wells. All peptides were prepared as a 4 mg/ml stock solution in phosphate-buffered saline. Peptide 3 (see above) required a 10-fold molar excess to inhibit the binding of CD59 to its ligand C8.

Mutational Analysis of CD59—Human to rat amino acid
substitutions that result in acquisition of rat complement inhibitory activity will identify functionally important residues. To determine whether the candidate residue groups identified by model comparison above are involved in the species selective function of human CD59, the groups of residues were substituted for corresponding rat residues. Some additional residues were also substituted so that all nonidentical residues within the 40–66 sequence were accounted for. Further, some substitutions were made for residues that are outside of the 40–66 sequence, but that neighbor human residues previously identified as important for CD59 activity (8, 9). The mutant human CD59 proteins containing groups of substituted rat residues that were initially prepared and tested are shown in Fig. 3 (mutant series A). The proteins were recombinantly expressed.

**Fig. 2. Diagrams of human and rat CD59.** Panels A and B show a comparison of molecular surfaces in the region identified as important for species selective function (residues 40–66). The surface of regions 1–39 and 66–77 that are not important for species selectivity is colored white. Conserved residues previously identified as functionally important in human CD59 (8, 9) are colored magenta and are not numbered. Side chains of all nonidentical residues within the 40–66 sequence (and potentially responsible for species selective activity) 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 therefore not important for species selectivity, are shown in white. Panel C is a ribbon diagram of human CD59 showing the residues experimentally determined to influence species selectivity.
on the surface of CHO cells, and cell populations expressing similar levels of protein were isolated (see “Experimental Procedures”) and then assayed for their susceptibility to human and rat serum. CD59 expression levels were quantitated using flow cytometry by means of an epitope tag inserted at the N terminus of all recombinantly expressed proteins as described previously (8, 11).

The data in Fig. 4 show that human CD59, rat CD59, and all chimeric CD59 proteins are equally effective against human complement, indicating that none of the substitutions had any adverse effect on protein conformation and activity. When compared with the activity of human CD59, the A1, A3, A6, and A7 chimeric proteins provided enhanced protection against rat complement. The A6 and A7 proteins were about 25 and 70% as effective as rat CD59 against rat complement, respectively (calculated based on the difference between rat complement-mediated lysis of CHO cells expressing either human or rat CD59) (Fig. 4). The A6 and A7 proteins were significantly more effective against rat complement than the A1 and A3 proteins. Each of the A6 and A7 chimeras contained only two substituted residues (Fig. 3), putatively identifying one or more human residues from a total of four that primarily determine the species selective activity of human CD59, i.e. Phe-47, Thr-51, Arg-55, and Lys-65. The human to rat R55E substitution is common to the A1, A3, and A6 proteins, suggesting that this substitution is responsible for the slightly increased inhibitory activity against rat complement of the A1 and A3 proteins.

In a second series of mutations, each of the four candidate functionally important human residues, and a residue not expected to effect species selectivity (Lys-41), were individually substituted for corresponding rat residues (see Fig. 3, series B). The data in Fig. 5 show that the individual substitution of each candidate human residues with the corresponding rat residue produced a protein with enhanced rat complement inhibitory activity. The F47A substitution (B4 mutant) was by far the most effective at enhancing the activity of human CD59 against rat complement. This single rat residue substitution in human CD59 resulted in a protein that was about 65% as effective as rat CD59 against rat complement (Fig. 5). The T51L and R55E substitutions resulted in proteins that each possessed close to 20% of rat CD59 inhibitory activity. The K65Q substitution also appeared to display a small (about 10%), but statistically insignificant increase in activity against rat complement. Nevertheless, the A6 protein that contains both an R55E and K65Q substitution (Fig. 4) was slightly more effective against rat complement than an R55E substitution alone (Fig. 5). The single human to rat residue substitution at position 41 (K41R) did not alter the functional characteristics of human CD59, as predicted from functional data obtained with the A2 protein (contains a K41R substitution). To further confirm an important role for residue 47 in determining the species selective function of human CD59, an additional mutant protein was prepared containing a human to mouse substitution at residue position 47 (protein B5 (F47G), see Fig. 3). We have shown previously that human CD59 is not effective against mouse complement (22), and the single F47G substitution produced a mutant protein possessing species selective function that was quantitatively similar to the F47A (human to rat) substitution (Fig. 5). None of the residue substitutions had any effect on human complement inhibitory activity, indicating that all recombinant proteins were correctly folded (Fig. 5).

The positions and side-chain characteristics of the identified functionally important human residues on the CD59 protein are shown in Fig. 2. Interestingly, the side chains of the Phe-47, Thr-51, and Arg-55 residues are all located in a strip on the same face of the CD59 helix (Fig. 2C). In a final mutant CD59 protein, each of the three human helix residues that individually affected species selective function were substituted with rat residues (mutant B7, Fig. 5). The rat complement inhibitory activity of this mutant protein approached that of rat CD59 (about 80% as effective) (Fig. 5), further indicating that the identified helix residues, and in particular Phe-47, are the principal determinants of human CD59 species selective function.

Effect of Synthetic CD59 Peptides on the Binding of CD59 to C8—We used a previously characterized microtiter plate binding assay to determine whether synthetic human CD59 peptides from the vicinity of the helix region could interfere with the binding of CD59 to its ligand, C8. We found two peptides, both spanning the helix residue sequence, that modestly inhibited the binding of human CD59 to human C8; a peptide to

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**Figure 3. Human to rat amino acid substitutions made in chimeric CD59 proteins.** The individual residue substitutions shown in series B were selected based on functional data obtained from series A mutant proteins.

**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 (panel A) or rat serum (panel B) and lysis percentage determined. A1–A7 represent mutant human CD59 proteins containing groups of rat residue substitutions (refer to Fig. 3). An N-terminal epitope tag that does not effect CD59 function was used to measure cell surface expression of CD59 (8, 11) (also see “Experimental Procedures”). Mean ± S.D. (n = 6).
CD59 ratio of 320:1 inhibited binding by 30–40% (peptides 2 and 3, Fig. 6). In contrast, the same peptides were much less effective at inhibiting the binding of rat CD59 to rat C8. This data is consistent with the above mutagenesis data and suggests that the identified CD59 helix residue(s) are directly involved in the species selective binding of C8. A shorter peptide containing the helix residues (peptide 4, Fig. 6), as well as a peptide containing sequence C-terminal to the helix residues (peptide 1, Fig. 6), had little effect on the binding of CD59 to C8. A possible explanation for the lack of inhibition by the short helix peptide is that the N- and/or C-terminal helix residue extensions of the longer peptides stabilize a structure that is more favorable for binding. In a functional assay, the CD59 peptides were also tested for their effect on human C5b-9-mediated hemolysis of human erythrocytes (as described in Ref. 23). Peptide binding to C8 in the assembling C5b-9 complex at a cell surface might interfere with the inhibitory effect of CD59, but the peptides had no effect on C5b-9-mediated hemolysis (not shown).

**DISCUSSION**

By measuring the inhibitory activity of chimeric human-rat CD59 proteins against human and rat complement, it was previously determined that the species divergent 40–66 residue sequence contains the residues important for the species restricted function of human CD59 (11). We have now identified individual residues within this region that are responsible for human CD59 species selectivity. Individual substitutions of the human residues Phe-47, Thr-51, and Arg-55 for corresponding rat residues, each produced proteins with enhanced activity against rat complement. The substitution of all three residues in a single protein resulted in a CD59 protein (termed B7) with a species selective activity that was quantitatively similar to that of rat CD59; compared with the negligible activity of human CD59 against rat complement, the B7 mutant was about 80% as effective as rat CD59 at inhibiting rat complement. A fourth residue, Lys-65, also appears to contribute to the selectivity of human CD59 function, albeit to a lesser degree than the three helix residues, and may at least partly account for the slightly reduced activity of the B7 protein against rat complement as compared with rat CD59. No other nonconserved residue within the 40–66 sequence had any detectable effect on the species selective function of human CD59.

The residues identified here as determinants of species selectivity are distinct from previously identified human CD59 active site residues. Site-directed mutagenesis of human CD59 (nonconservative substitution) has indicated that residues Phe-23, Asp-24, Trp-40, Arg-53, Leu-54, Glu-56, and Tyr-62 are important for human CD59 function (8–10). With the exception of Tyr-62, these residues are located on the membrane-distal face of CD59 in the vicinity of a hydrophobic cleft, and with the further exception of residues Phe-23 and Glu-56, they are conserved in human and rat CD59 (refer to Fig. 1). It is possible that CD59 from different species possess a conserved ligand binding site, and that nonconserved residues in CD59 proteins influence the specificity of ligand binding via indirect or allosteric mechanisms. Alternatively, CD59 residues involved in determining the species selectivity may participate directly in ligand binding. We identify Phe-47, Thr-51, and Arg-55 as being the residues primarily involved in restricting human CD59 activity. In rat CD59, these residues are replaced by alanine, leucine, and glutamic acid, respectively. Interestingly, these three residues form a continuous strip parallel to the axis of the CD59 helix and are exposed to the solvent. It is therefore considered unlikely that the side chains of residues Phe-47, Thr-51, and Arg-55 influence specificity through affecting the relative position of the helix with respect to other binding pocket residues. Rather, it seems more likely that these residues are directly involved in ligand binding. The side-chain differences between the three human and rat CD59 residues are such that all three substitutions can potentially contribute to specificity, provided that this phase of the short helix is involved in the direct interaction. The phenylalanine and alanine side chains are both hydrophobic but differ in size, the threonine and leucine differ by a polar group and hydrophobic character, and arginine and glutamic acid have different charges although they share the same hydrophobic stem.

The single residue that contributes by far the most to human CD59 species selective function is Phe-47. A key role for Phe-47 in species selectivity was further indicated by functional analysis of a human to mouse F47G substitution (see “Results”). Perhaps a binding pocket on the rodent C8/C9 ligands that can accommodate the rodent alanine and glycine residues cannot accommodate the large phenylalanine residue in the corresponding location on human CD59. Such an explanation is compatible with the fact that rat (11) and mouse (22) CD59 are both effective against human complement, whereas human CD59 does not function effectively against rodent complement. Also compatible with this “docking” concept is the previous result that a nonconservative F47E mutation resulted in a human CD59 protein with only a weak protective effect against human complement (9). It was suggested from this finding that Phe-47 may be at the periphery of the human CD59 active site. The positively charged residue Lys-65 in human CD59 that is replaced by a polar Gln-65 in rat CD59 also had a small effect on species selectivity. Lys-65 is positioned next to the conserved and functionally important residue Asp-24 (9) and is located at one end of the hydrophobic cleft that may be important for complement ligand binding (see above). The current data does not exclude the possibility that the location of residues that determine species selectivity may differ in different CD59 proteins. Nevertheless, consistent with the current data, a recent analysis of the species selectivity of chimeric human/
animal models, and an understanding of the molecular basis for CD59 function may provide the rationale for the design of efficient soluble MAC inhibitory constructs for clinical application. Inhibiting the terminal pathway of complement but leaving the activation pathway intact may offer significant clinical advantages in diseases in which the MAC plays an important role. This is because products of the complement activation pathway play important roles in immunity to infection and in immune complex catabolism. Evidence indicates that an effective CD59-based inhibitor will also provide efficient protection from complement-mediated hyperacute rejection of xenotransplanted tissue (24, 25). Transgenic pig organs expressing high levels of human CD59 are protected from human complement and show prolonged survival when transplanted into primates (26, 27). The identification here of the residues important for species selectivity and the conservation of these residues in human and pig CD59 support the view that the level of CD59 expression will be more important than the species of CD59 in prolonging pig to human graft survival (28, 29). Finally, defining the functional site(s) of CD59 may also assist in the design of inhibitors of CD59. Inhibiting CD59 function on the surface of tumor cells may prove effective in anti-tumor complement-dependent immunotherapy.

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