

Structure-Function Relationships of the Complement Regulatory Protein, CD59

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ABSTRACT: CD59 (membrane inhibitor of reactive lysis, protectin) is a membrane protein whose functions include the inhibition of the insertion of the ninth component of complement into the target membrane. It belongs to a superfamily of proteins including Ly-6, elapid snake venom toxins, and urokinase receptor (UPAR); the members of the superfamily have a similar structure that includes four (in mammals five) disulfide bridges that maintain a three-dimensional conformation consisting of a central core, three finger-like “loops” extending from it and a small loop near the carboxyl end. We have used site directed mutagenesis to explore three aspects of the structure of CD59: 1) the role of the disulfide bridges in expression and function of the molecule; 2) the location of epitopes reacting with monoclonal antibodies to the molecule; and 3) the parts of the molecule that are critical to its function in inhibiting complement lysis. Mutant molecules in which the disulfides maintaining the finger-like loops (Cys3-Cys26, Cys19-Cys39, and Cys45-Cys63) were removed were not expressed on the cell surface. The mutation of the disulfide (Cys6-Cys13) resulted in no change in expression or function. The mutation of Cys64-Cys69 maintaining the small loop resulted in an expressed molecule with increased functional activity. The major epitope for 6 of 7 monoclonal antibodies was centered on Arg53 as the mutation 53Arg→Ser resulted in a loss of interaction with these antibodies, as did the deletion of four nearby residues (Leu54-Asn57). The alteration 55Arg→Ser resulted in loss of reactivity for some but not other antibodies. The reactivity with one monoclonal antibody, H19, was abrogated by the mutations 61Tyr→Gly and 61Tyr→Ala. Functional activity of the molecule was not adversely altered by mutations in the first and second loops; however, the 61Tyr→Gly mutation was non-functional. The mutation of 61Tyr→His diminished function but changes 61Tyr→Ala and 61Tyr→Phe had no effect on function. We conclude that the functional site of CD59 is located in this region of the molecule.

Keywords: CD59, mutation, function, structure

INTRODUCTION

D59 is a membrane glycoprotein with a molecular weight of 19 kD that inhibits the cytolytic action of complement (1-3) and interacts with other proteins of T lymphocytes (4-6). The comple-

ment-inhibitory function of CD59 derives from its capacity to interact with the C8 and C9 components of the cytolytic membrane attack complex to block the conversion of C9 from a hydrophilic to an amphipathic molecule, thus preventing insertion of C9 into the membrane (2,3,7). Transfection

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of non-primate cell lines with the cDNA for human CD59 confers species-selective complement resistance to lysis by the human C5b-9 complex (8).

Human CD59 is composed of 77 amino acids and contains 10 cysteine residues that form five intramolecular disulfide bonds (9-11). At least some of the disulfide structure is necessary for CD59 function since reduction and alkylation results in its inactivation (12). Inactivation in this way is almost certainly the reason for the increase in complement sensitivity of normal human red cells when treated with aminoethyliso-thiouonium bromide (AET) (13) or 2-mercapto-ethanol (14). The molecule also contains one glycosylation site (residue Asn18) (15) and is attached to the membrane by the glycosyl-phosphatidylinositol (GPI) anchor (16).

CD59 belongs to a family of molecules characterized by evolutionary conservation of the arrangement of the disulfide bridges. Members of this family include the murine protein, Ly-6, (17,18) and the urokinase-plasminogen activator receptor (UPAR) (19); in the latter molecule, there are two exact and one inexact repeats of the cysteine-related structure of the molecule. This complex cysteine-rich structure has also been found in a number of elapid snake venom toxin molecules (20), a squid protein (21) and in a CD59-like membrane protein of herpes virus Saimiri (22). Evidence that these diverse proteins are all part of a structural superfamily is also found in the similarity of the organization of the genes encoding these molecules. In each case, the coding region consists of an exon coding for most of the leader peptide sequence, a short exon coding for the first one-third of the mature protein, and a third longer exon coding for the rest of the polypeptide (17,18,23); in UPAR, the short exon and longer exon are repeated twice more before the sequence coding for the attachment to the anchor occurs (24).

The three-dimensional structure of CD59 has recently been elucidated by NMR analysis (25,26). It is similar to that of the snake venom toxins

including erabutoxin, bungarotoxin, cardiotoxin and others (27-30). These proteins share a canonical structure consisting of a central hydrophobic core with three finger-like “loops” held by disulfide bridges extending from it. A fourth loop, near the carboxyl terminus, is always small (four or five residues) and its distal cysteine is always followed by an arginine residue. CD59 is unique among the members of the Ly-6 superfamily whose structure is known in that it contains a helical structure in the third and largest loop (25).

We have used site-directed mutagenesis to investigate three aspects of this structure: 1) the specific requirement for each intrachain disulfide in the cellular expression and complement-inhibitory function of CD59, 2) the segments of the CD59 polypeptide that contribute to the epitopes recognized by seven monoclonal antibodies reacting with this molecule and 3) the residues within CD59 that are critical for normal expression of its complement-inhibitory function.

METHODS

Site-Directed Mutagenesis by PCR

The template for mutagenesis was a 463 bp CD59 cDNA to which *EcoRI/HindIII* adapters were ligated, and which was then inserted into the polylinker *HindIII* site of expression vector pRc/RSV (Invitrogen Corp.). Specific residues were modified using PCR-based oligonucleotide-directed mutagenesis (31). The first round of PCR amplification was primed by one of a pair of complementary mutagenized oligonucleotides, along with an oligonucleotide primer upstream (5'GCTCGATACAATAAACG3') or downstream (5'GAAGGCACAGTCCAGGC3') from the pRc/RSV polylinker. The resulting PCR products were isolated by electrophoresis on a 2% low melting point agarose gel and recovered using Magic PCR Preps (Promega Corp.). The second PCR reaction

was primed via overlap extension of the first products, as well as by the upstream and downstream primers in the vector. The resulting product was gel purified as described above and digested with *HindIII*. It was then re-cloned into pRc/RSV which was used to transform *E. coli* strain TG-1 cells. All mutagenized cDNA constructs were sequenced in their entirety to verify the presence of the desired mutations as well as the fidelity of the remainder of the construct.

Transfection

Chinese hamster ovary (CHO) cells were transfected either with expression vector pRc/RSV alone (mock), or with the vector containing wild type or mutagenized CD59 cDNA. CHO cells were harvested by trypsinization and replated at 5×10^5 cells per 100 mm tissue culture dish (Costar) in 10 ml Alpha MEM medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco). Cultures were incubated for 18 h after which the old medium was aspirated and replaced with 3 ml of fresh medium plus 2 μ g transfecting DNA and 3.0 μ l of polybrene (Sigma) solution (10 mg/ml in water, filter-sterilized). After incubation for 6 h at 37 C, the DNA-containing medium was aspirated and 5 ml of Alpha MEM plus 30% DMSO (Fisher) was added for 2 min. The transfected cells were washed with 10 ml of serum-free medium which was replaced with 10 ml of Alpha MEM + 10% fetal bovine serum. Cells were incubated for 48-72 h, then replated in selective medium (Alpha MEM + 0.5 mg/ml geneticin (Gibco)). The medium was replaced with fresh selective medium every 3-4 days until the emergence of resistant colonies at approximately 2 weeks.

Detection of CD59 and Cloning of Transfected CHO Cells

Transfected CHO cells were trypsinized and incubated with a panel of monoclonal antibodies

[MEM-43 (SanBIO, AmUDEN, Holland), YTH 53.1 (Serotec Ltd. Oxford), H19 (kind gift of Dr. Alain Bernard, INSERM V, Nice, France), 10G10 (kind gift of Dr. Marilyn J. Telen, Duke University), 1F5, 1F1 (kind gift of Yasuko Nakano, Showa University, Tokyo), 2/24 (New South Wales Hybridoma Laboratory, Australia)] and polyclonal antibodies to CD59. Cells and antibodies were incubated for 30 min. at 4° C and excess antibody was removed by washing with PBS + 1% BSA. FITC-conjugated secondary antibodies (goat anti-mouse IgG/FITC (Tago, Burlingame CA) for MEM-43, H19, 10G10, 1F5, 1F1 and 2/24; goat anti-rat IgG/FITC (Tago, Burlingame, CA) for YTH; goat anti-rabbit IgG/FITC (Jackson ImmunoResearch, West Grove, PA) for polyclonal anti-CD59) were then added, allowed to incubate for 30 min. at 4° C, and the excess removed by washing with PBS + 1% BSA. The cells were then fixed with .05% formalin and analyzed using a PROFILE fluorescent activated cell sorter (Coulter, Hialeah, FL). Positive transfectants (those expressing CD59) were cloned by limiting dilution at 0.5 cells per well in 96 well plates.

Northern Analysis of Non-expressing CHO Transfectants

Northern blotting was used to detect CD59 transcripts in those CHO transfectants not expressing CD59 on the cell surface. Ten micrograms of total RNA were subjected to denaturing electrophoresis using an 18% formaldehyde, 1.2% agarose, MOPS buffer gel. After overnight capillary transfer to Duralon-UV nylon membranes (Stratagene Corp.), the membranes were cross-linked with ultraviolet light and prehybridized in 5.5 ml of QuickHyb solution (Stratagene Corp.) for 1 h at 68° C. Approximately 100 ng of the 463 bp CD59 cDNA was labeled with [γ^{32} P]dCTP using a random-primed labeling kit (Boehringer Mannheim). The labeled probe plus 1 mg of sheared herring testis DNA (Sigma) were dena-

tured by boiling for 5 min., added to the prehybridizing membrane, and allowed to hybridize at 68° C for 1.5 h. The membrane was washed twice at 20° C and once at 50° C in 300 mM NaCl, 30 mM Sodium citrate, 0.1% SDS, 2 mM EDTA, pH 7.0.

ELISA of Non-expressing CHO Transfectants

CHO transfectants not expressing CD59 on the surface were assayed for intracellular CD59 pools by ELISA. Cells were washed twice in Dulbecco's PBS and then lysed in TBS buffer [20mM Tris, 150 mM NaCl + 0.03% Triton X-100 (Sigma)], followed by sonication for 15 sec. Total protein was determined by the Bradford Dye-binding procedure (Bio-Rad), and samples diluted to 1, 10, or 100 µg/100 µL in TBS. Triplicates (100 µL) of each dilution were applied to high-binding 96-well plates and allowed to bind overnight at 4° C. The lysate was aspirated, and each well was washed x2 with TBS + 0.5% Tween 20 (TBST), and was incubated with blocking solution (TBS + 3% bovine serum albumin + 0.02% NaN₃) for 2 h at room temperature (RT). Blocking solution was discarded and 50 µL of polyclonal anti-CD59 or normal rabbit serum (1:1000) was applied for 1.5 h at RT. Wells were washed x3 in TBST, incubated with 1:2000 alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) for 1 h at RT, then washed x2 with TBST and x1 with TBS. 50 µL of substrate (1 mg/ml p-nitrophenyl phosphate + 1 mM MgCl₂ in Tris buffer, pH 9.8) was added and the plates read immediately on a V Max automated plate reader (Molecular Devices).

Assay for C5b-9-inhibitory Activity

The functional activity of recombinant CD59 was evaluated on the basis of the C5b-9-dependent release of previously-incorporated cytoplasmic 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxy-

fluorescein, acetoxymethyl ester (BCECF-AM) using methods described in Zhao *et al.* (8). Briefly, CD59-transfected CHO cells were grown to near confluence (80% < confluence < 95%) in 48-well culture dishes. The cells were briefly washed with HBSS containing 1% w/v bovine albumin and incubated (30 min., 37° C) with 15 µM BCECF-AM. The cells were washed free of unincorporated dye and incubated (30 min., RT) with 3 mg/ml rabbit antibody against CHO plasma membrane, after which C5b-9 assembly was initiated by incubation (15 min., 37° C) with 25% v/v C8-deficient human serum (C8D) supplemented with 0-1 µg/ml human C8. The C5b-9-dependent release of BCECF was determined by measurement of supernatant and cell fluorescence, with correction for non-specific release from controls omitting C8. In some experiments, C5b67 was first deposited by incubation (20 min., 37° C) with 25% v/v C8D, the C5b67 cells washed free of serum, and incubated with either purified human C8 and C9 or EDTA-chelated serum (5% v/v human or guinea pig) to complete assembly of the membrane attack complex. Following 15 min. incubation at 37° C, the C5b-9-dependent release of BCECF was determined by measurement of supernatant and cell fluorescence, with correction for non-specific release from controls omitting C8 and C9. Total cell-associated fluorescence was determined by lysis in 10% v/v sodium dodecyl sulfate. BCECF fluorescence was measured using an SLM 8000C fluorimeter in the photon counting mode with 490 nm and 530 nm excitation and emission wavelengths, respectively.

RESULTS

The Effect of Mutagenesis of One or Both Members of Each Disulfide Bridge In order to examine the role of the disulfide bond structure in the expression and function of CD59, we sequentially mutated the cysteines of each of the disulfide pairs (Cys19-Cys39, Cys3-Cys26, Cys45-Cys63,

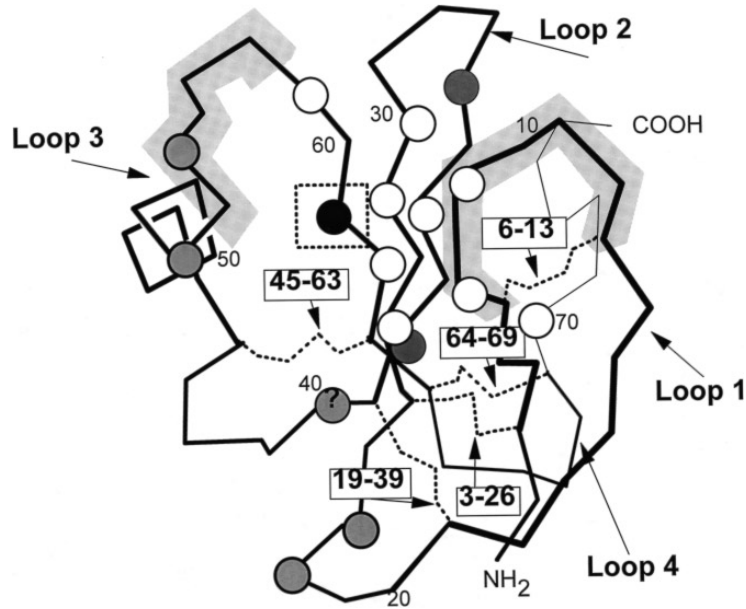


Figure 1. Model of the structure of CD59 derived from NMR studies of Fletcher *et al.* (25) showing sites of mutation and deletion. The disulfide bridges are indicated by light dotted lines and are numbered by the two cysteines involved. The open circles indicate sites at which mutagenesis did not affect the functional capacity of the molecule. Mutagenesis at the sites indicated by black symbols resulted in alteration in functional activity. The symbol surrounded by a dashed square indicates Tyr61; mutagenesis of this residue to Gly resulted in a molecule without function whereas mutagenesis to His or Ala diminished function slightly and change to Phe did not alter function. Mutagenesis of Gln34 and Lys38 increased functional activity of the molecule. Mutagenesis of residues at residues with cross-hatched symbols resulted in altered interaction with 6 of 7 monoclonal antibodies. The residues deleted in loop 1 and loop 3 are shown in shaded area.

Cys64-Cys69, and Cys6-Cys13) (see Figure 1). When constructs were made in which a single member of each cysteine-cysteine disulfide pair was changed to serine, no CD59 protein was detectable on the surface of the transfected cells by any of 4 monoclonal (MEM-43, 10G10, YTH 53.1, and 1F5) or polyclonal anti-CD59 antibodies. In each case, mRNA specific for CD59 of the appropriate size and signal strength was detected by Northern blotting in the transfected cells but not in the untransfected cells, indicating that the transfected cDNA had been transcribed (data not shown). No protein could be detected by ELISA of whole cell lysates using polyclonal anti-CD59 antibodies (data not shown). Similarly, no surface or intracellular CD59 could be detected in transfectants in which the disulfide pairs Cys19-Cys39, Cys3-

Cys26, or Cys45-Cys63 were eliminated by mutation of both cysteines to serines. In each case, CD59-specific mRNA was found in the transfected cells.

By contrast to the negative results described above, mutagenesis to eliminate both of the cysteines of the disulfide internal to loop 1 (Cys6-Cys13) or of the disulfide forming loop 4 (Cys64-Cys69) resulted in immunoreactive CD59 molecules that were expressed on the cell surface as shown by reaction with monoclonal and polyclonal antibodies. No change in complement-inhibitory function was observed for mutant CD59 in which the disulfide bond internal to loop 1 (Cys6-Cys13) was disrupted by Cys-to-Ser mutation. By contrast, mutation to disrupt the disulfide bond joining loop 4 (Cys64-Cys69) resulted in a small but significant increase in the

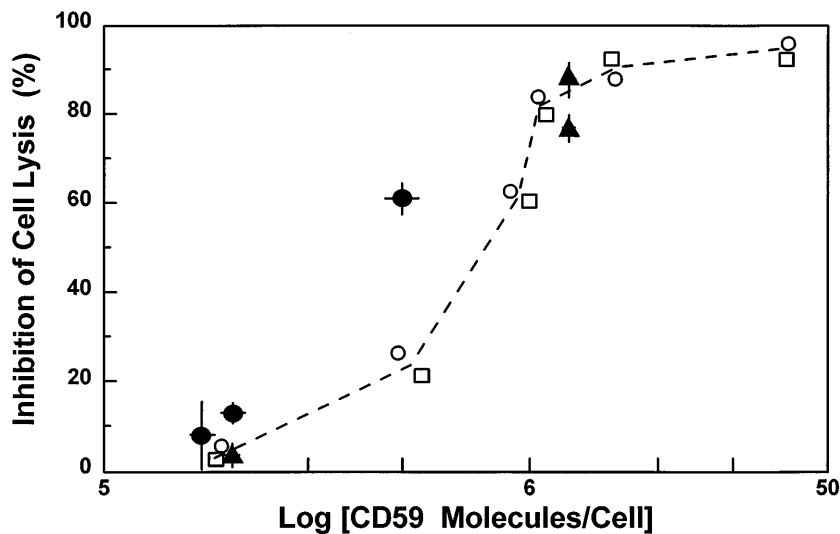


Figure 2. The effect of eliminating the disulfide bridges holding loop 1 (Cys6 and Cys13 changed to Ser6 and Ser13) (solid triangles) and loop 4 (Cys64 and Ser64 changed to Cys69 and Ser69) (solid circles) on the ability of CD59 to inhibit the lysis of CHO cells by human C5b-9. The dose-response curve for unchanged CD59 (wild type) is shown for comparison (open symbols). The lines through the symbols indicate ± 1 SD

complement-inhibitory function of CD59 (Figure 2).

The Effect of Mutagenesis on Epitope Expression

In order to identify the segments of polypeptide contributing to the epitopes recognized by available CD59-reactive monoclonal antibodies, various mutations were introduced into CD59, the mutant proteins expressed in CHO cells, and antibody binding determined (Table 1). CD59-reactive monoclonal antibodies analyzed included MEM-43, YTH 53.1, 1F5, 1F1, 2/24, 10G10, and H19, and results are summarized in Figure 3.

These studies suggest that the epitope necessary for recognition by all but one (H19) of these monoclonal antibodies is centered on Arg53, with a contribution by residues Leu54-Asn57. In the case of each mAb tested (except H19), the alteration or deletion of these residues in CD59 resulted in the loss of reactivity with antibody. Whereas the epitopes of each of these

monoclonal antibodies appeared to be principally determined by residues flanking Arg53, a contribution of other segments of CD59 to the epitope of each of these antibodies was also apparent; for example, mutation 55Arg→Ser and the more distant 22Asp→His + 24Arg→His resulted in variable loss of antibody binding, although the impact of such mutations on epitope expression varied widely with each antibody and may therefore reflect steric or non-specific effects.

By contrast to the other monoclonal antibodies, the mutations described above had no effect on the binding of antibody H19. Two mutations at Tyr61 (61Tyr→Gly and 61Tyr→Ala) resulted in complete loss of H19 binding, whereas binding of the other 6 monoclonal antibodies was unaffected by these mutations. Mutation of Tyr61 to Phe61 or His61 resulted in a mutant molecule that reacted normally with H19 and all other monoclonal antibodies. The experiments therefore suggest that H19 binds to an epitope of CD59 that depends upon the phenolic ring of Tyr61 or its hydrophobicity, and that the H19 epitope is distinct from the

Table 1. Expression and Function of Site-Directed Mutants of CD59

Mutation	Expression by Flow Cytometry Using Anti-CD59			Function (Inhibition of C5b-9 Lysis)
	Monoclonal		Polyclonal	
	Others ¹	H19		
Disulfide Bridges				
Cys 3, Cys 26 → Ser 3, Ser 26	No	NT	No	NA
Cys 6, Cys 13 → Ser 6, Ser 13	Yes	NT	Yes	Normal
Cys 19, Cys 39 → Ser 19, Ser 39	No	NT	No	NA
Cys 45, Cys 63 → Ser 45, Ser 63	No	NT	No	NA
Cys 64, Cys 69 → Ser 64, Ser 69	Yes	NT	Yes	Increased
Other Mutations and Deletions				
Pro 7 → Ala 4	Yes	Yes	Yes	Normal
Pro 9 → Ala 9	Yes	Yes	Yes	Normal
Deletion Pro 7—Cys 13	Yes	Yes	Yes	Normal
Asp 22, Asp 24 → His 22, His 24	Altered ²	Yes	Yes	Not Done
Leu 27 → Val 27	Yes	Yes	Yes	Normal
Thr 29 → Ser 29	Yes	Yes	Yes	Normal
Lys 30 → Leu 30	Yes	Yes	Yes	Normal
Gln 34 → Leu 34	Yes	Yes	Yes	Increased
Tyr 36 → His 36	Yes	Yes	Yes	Normal
Lys 38 → Glu 38	Yes	Yes	Yes	Increased
Trp 40 → Asp 40	No	No	No	NA
Trp 40 → Leu 40	No	No	No	NA
Trp 40 → Phe 40	Yes	NT	Yes	? Decreased
Arg 53 → Ser 53, Arg 55 → Ser 55	No	Yes	Yes	Normal
Arg 53 → Ser 53	No	Yes	Yes	Normal
Arg 55 → Ser 55	Altered ²	Yes	Yes	Normal
Deletion Val 50—Thr 60	No	No	No	NA
Deletion Leu 54—Asn 57	No	Yes	Yes	Normal
Leu 59 → Met 59	Yes	Yes	Yes	Normal
Tyr 61 → Gly 61	Yes	No	Yes	None
Tyr 61 → Phe 61	Yes	Yes	Yes	Normal
Tyr 61 → Ala 61	Yes	No	Yes	Slightly Decreased
Tyr 61 → His 61	Yes	Yes	Yes	Decreased
Tyr 62 → Asp 62	Yes	Yes	Yes	Normal
Asn 70 → Gln 70	Yes	Yes	Yes	Normal

¹ Including 10G10, MEM-43, YTH 53.1, 1F1, 1F5, and 2/24 (see text).² The reaction of this mutant varied with different monoclonal antibodies (see text).

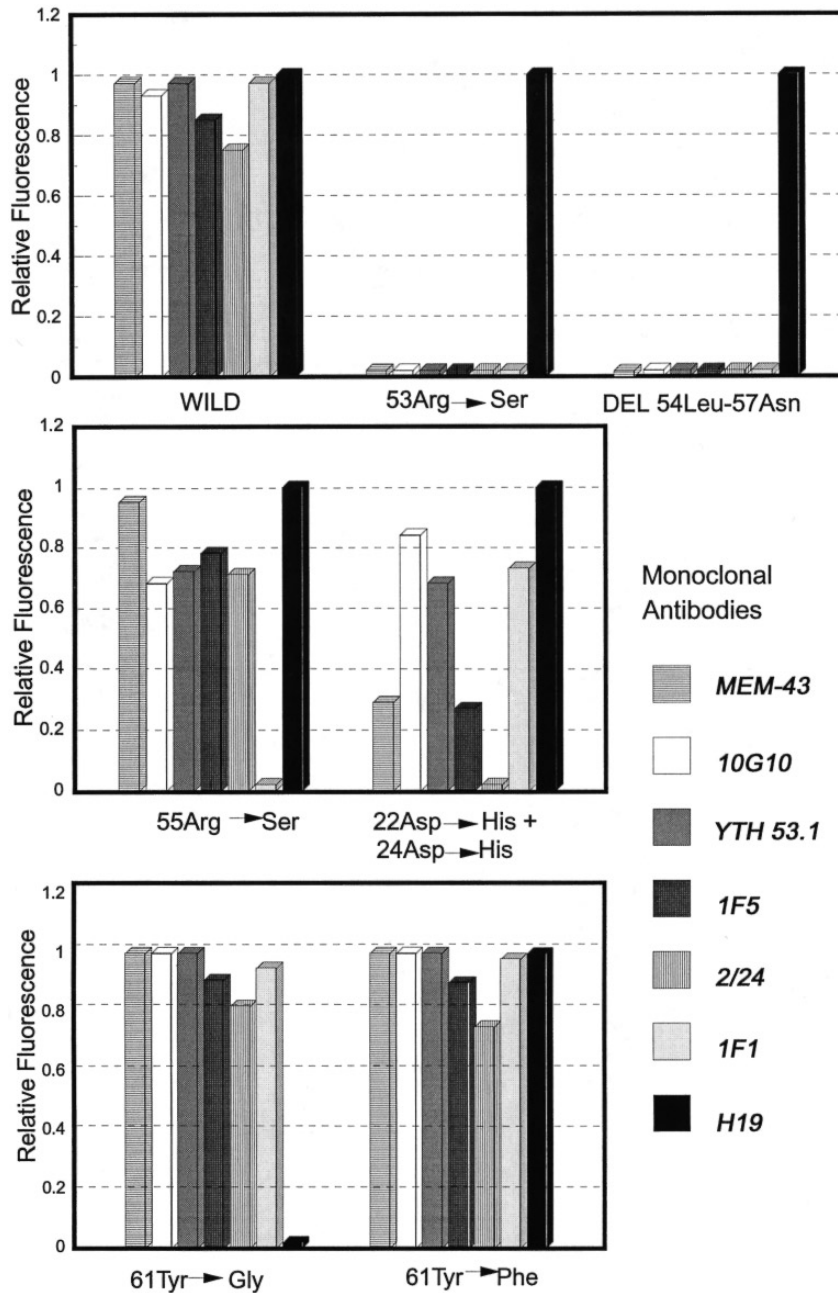


Figure 3. The reactivity of 7 monoclonal antibodies against unmutated CD59 (wild) and various mutants. The mean channel fluorescence of H19 on wild type was taken as a relative fluorescence of 1.0. Clones were selected that expressed approximately the same number of molecules of CD59. Only those mutations showing differences in reactivity are shown.

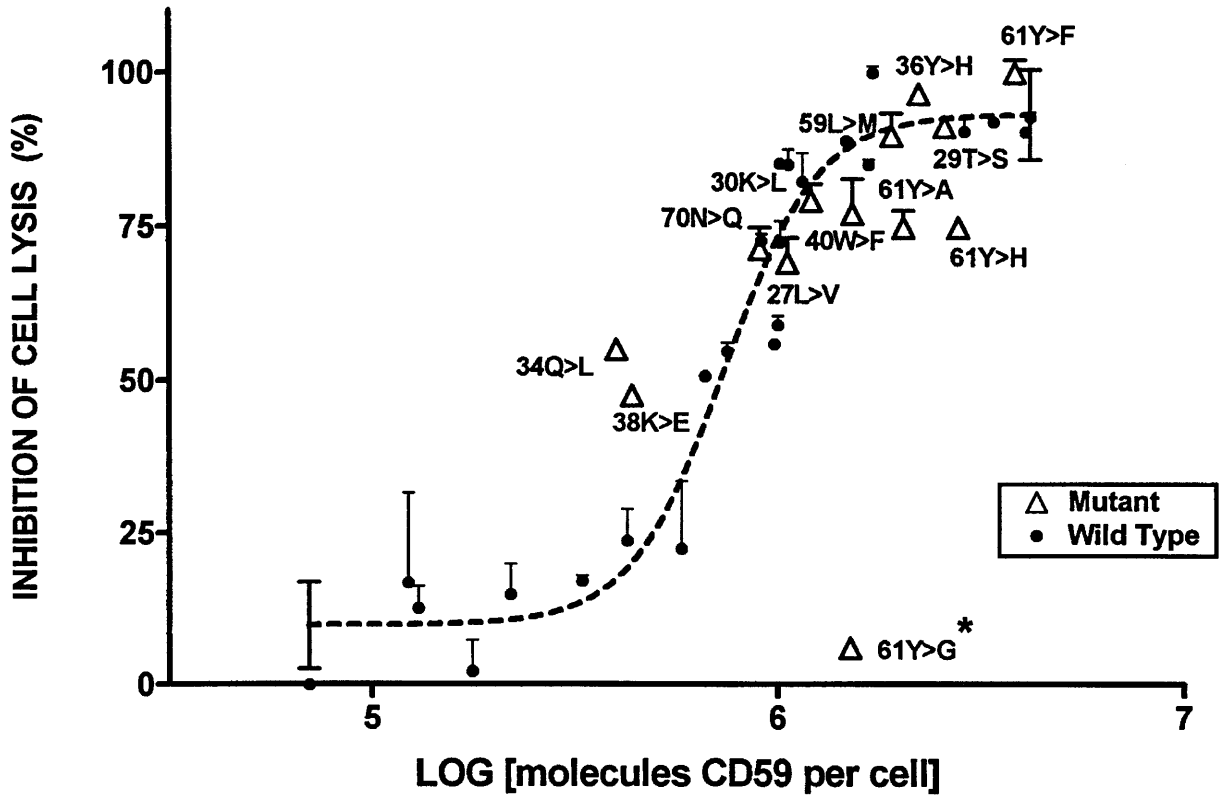


Figure 4. Functional activity of CD59 mutants expressed in CHO cells. Capacity of cells to resist lysis by human complement (ordinate) was determined by BCECF dye release assay, and is expressed as percent inhibition of cell lysis relative to mock-transfected CHO controls. Abscissa indicates number of molecules of CD59 expressed on the cell surface, determined from binding of mAb 10G10 (see *Materials & Methods*). Data for Wild type CD59 transfectants are indicated by closed circles. Dashed curve indicates non-linear regression of all data obtained in multiple assays of Wild type CD59 clones, with 95% confidence levels indicated by bold error bars. Data for CD59-mutants are indicated by *open triangles*, with amino acid substitutions indicated by single letter code. Error bars associated with data for individual clones denote SD. Asterisk indicates 61Tyr→Gly mutation.

overlapping epitopes recognized by all other CD59-reactive monoclonal antibodies.

The Effect of Mutagenesis on Complement Inhibitory Function

The residues to be mutagenized or deleted were selected on the basis of computer modeling of the molecule of CD59 and by reference to the published structure (25,26). In each case, the mutant protein was expressed in cDNA-transfected CHO cells, the cells were cloned to obtain individual lines expressing well-defined surface

concentrations of CD59 (wild type or mutant), and the relative complement-inhibitory activity of each expressed protein determined with reference to that of wild type CD59, based on the measured inhibition of fluorescent dye loss due to pore formation by the human MAC (see *Materials & Methods*). As summarized in Table 1 and Figure 4, no loss in the complement-inhibitory function of CD59 was detected upon alteration of residues contained within the segment of polypeptide forming either loop 1 (mutation of residues 7 and 9 and deletion of residues 7-13) or loop 2 (mutation of residues 27, 29, 30, 34, 36, and 38). Furthermore,

no loss of function was observed when mutations were introduced in the segment of loop 3 that contains α -helical structure (mutation of residues 49, 53, 55, 59, and 62 and elimination of residues 54–57), although this segment of the protein significantly contributes to the immunodominant epitope of the protein, shown to be recognized by 6 out of 7 monoclonal antibodies against CD59.

The tertiary structure of CD59 is remarkable for the presence of aromatic residues, including Tyr61 that are exposed to solvent on the surface of the protein, and it has been suggested that the face of the protein containing these hydrophobic residues might function in the interaction with the C5b-9 complex (32). As shown in Figure 4 and Table 1, a complete abrogation of the function of CD59 was observed upon substitution of Tyr61 by Gly61, such mutation resulting in a protein that was fully expressed but showed no detectable inhibitory activity against human MAC. By contrast to the loss of function observed with Tyr61→Gly61 mutation, the complement-inhibitory function of CD59 was retained when Tyr61 was replaced by His, Phe, or Ala, although such substitutions did cause a loss of reactivity with the anti-CD59 monoclonal antibody H19.

CD59 has previously been shown to exhibit relative species selectivity in its interaction with the membrane attack complex, with greatest inhibitory function observed when C8 and C9 derive from human and other primate serum (2,8). For example, whereas CD59 binds and inhibits the function of human C8 and C9 to prevent MAC assembly, CD59 neither binds nor inhibits the lytic activity of rabbit or guinea pig C8 and C9. In order to confirm the selective loss of CD59 function in CHO cell lines expressing the Tyr61→Gly mutation, we compared the susceptibility of wild type and mutant CD59 transfectants to lysis by MAC assembled using either human or guinea pig serum as the source of C8 and C9 (Figure 5). Recombinant wild type CD59, but not the Tyr61→Gly mutant, showed the capacity to inhibit the activity

of human C5b-9, whereas neither wild-type nor mutant CD59 inhibited lysis when guinea pig C8 and C9 were substituted for human C8 and C9 in the membrane attack complex. These data confirm that the complement-inhibitory activity attributed to wild type CD59 transfectants related to the capacity of CD59 to specifically restrict the lytic activity of human C5b-9, and was not due to an overall resistance of the CD59-transfected cell lines to lysis *per se*.

DISCUSSION

Amino acid sequence homology indicates that CD59 is related to the Ly-6 series of murine lymphocyte antigens (17), as well as the elapid snake venoms such as erabutoxin (27), bungarotoxin (33), cobratoxin (34), and cardiotoxin (35). Each of these proteins is characterized by a structure comprised of 4 internal disulfides that maintain the three-dimensional structure of a central hydrophobic core, three finger-like projections composed primarily of beta-sheet, and a fourth small loop behind this structure. Structural studies by NMR (25) have shown that CD59 is similar in structure to the well-described structure of the snake venom toxins but with several differences:

1. CD59, and all mammalian proteins of this superfamily for which structure is known, contain a disulfide (Cys6-Cys13 in CD59) that spans across the first loop. This bond is not found in the snake venom toxins and other non-mammalian members of the Ly6-superfamily.
2. CD59 and other known mammalian proteins of this superfamily have a long sequence at the carboxyl end of the molecule (residues 70–77 in CD59) by which it is bound to the cell surface through a glycosyl phosphatidylinositol (GPI) linkage.
3. CD59 has an α -helical structure in the third loop that is not present in other members of the

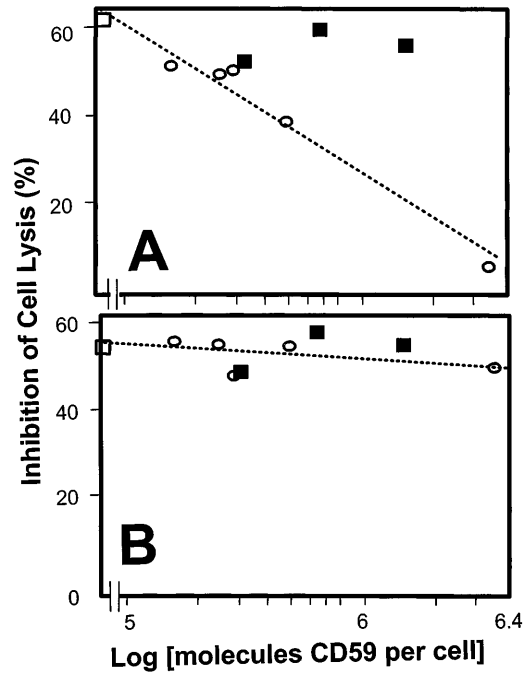


Figure 5. Loss of C5b-9 inhibitor function upon 61Tyr→Gly mutagenesis. Wild-type CD59 (open circles) and CD59 containing 61Tyr→Gly mutation (solid squares) were each expressed in CHO cells and the cells assayed for cell surface expression of CD59 antigen (*abscissa*) and sensitivity to lysis by complement (ordinate). Surface expression of CD59 antigen was measured by analysis of the binding of ^{125}I -labeled mAb 10GI0 (.see Methods). Cell lysis was determined from the release of intracellular BCECF in cells exposed to human CI-C7 and either human C8 and C9 (Panel A) or guinea pig C8 and C9 (Panel B). In each case, serum containing 10 mM EDTA served as source of C8 and C9, and correction was made for non-specific dye release observed in the absence of serum (see Methods). Open squares represent dye release measured for mock-transfected controls, lacking CD59. Dashed lines represent linear least square regression of corrected data for cells lines transfected with wild type CD59.

Ly-6 superfamily for which structure has been determined (25). This segment of α -helix partially covers the hydrophobic area associated with the central portion of the molecule.

4. CD59 contains a complex polymannosyl polysaccharide through N-glycosylation at residue Asn18 (15).

The present studies on CD59 demonstrate that three of the four fundamental disulfides (those holding the major “loops” of protein in place) are necessary for structural integrity of the molecule (Cys3-Cys26, Cys19-Cys39, and Cys45-Cys63); when both members of these bridges were altered to serine, no CD59 protein could be found on the surface or in the cytoplasm of the transfected cells

by immunochemical reactions, even though mRNA could be detected in the cytoplasm, indicating that the transfection was successful. These results suggest that each of these disulfides is required to either form or to maintain the stably-folded structure of the protein. It is somewhat surprising that in the case of CD59, disruption of the Cys45-Cys63 disulfide holding loop 3 should result in non-expression of cell-surface protein, since this disulfide bridge is not found in the first “CD59-like” repeat contained in the urokinase receptor (36).

The two other disulfides contained in CD59 [Cys6-Cys13 internal to loop 1 and Cys64-Cys69 forming loop 4) do not appear to be essential to structural integrity. Of note, the disulfide forming

loop 4 is very highly conserved in the Ly-6 protein superfamily, and is found in CD59, Ly-6, urokinase receptor, and all the snake venom toxins. It is therefore surprising that a disruption of this loop by cysteine mutagenesis should have no effect on CD59 expression.

The elimination of the disulfide internal to Loop 1 of CD59 (Cys6-Cys13) results in the expression of a protein with unaltered biological activity and with no detectable change in reactivity with the anti-CD59 monoclonal antibodies tested. This disulfide would appear to stabilize the structure of Loop 1, which may also be stabilized by interactions between the segments of polypeptide comprising the two arms of the loop. The results obtained with this mutant imply either that expression and reaction with the available antibodies do not depend upon the stabilized loop 1 structure, or, that side-chain interactions among residues forming the arms of loop 1 are sufficient to maintain three-dimensional integrity in the absence of the Cys6-Cys13 disulfide.

These studies suggest that there are two major epitopes on the CD59 molecule reacting with the seven monoclonal antibodies tested. The most antigenic appears to be that centered on Arg53 which is located in the α -helical structure of loop 3 (25); however, the epitope structure depends in part on surrounding moieties since elimination of four neighboring residues (Leu54-Asn57) also results in loss of antibody reactivity. Alterations in interaction of these six antibodies with the mutant 55Arg \rightarrow Ser55 can be explained by its proximity to this important epitopic area; reaction with one (MEM) was unaltered whereas reaction with 1F1 was abrogated by this change. However, the alterations in reactivity of the mutant 22Asp \rightarrow A His + Asp24 \rightarrow His are less easily explained because of the apparent distance of these residues from Arg53; in the NMR model of the molecule, the distance is probably too great to be part of the same antibody binding area (see Figure 1). This suggests that long-range interactions between these peptide regions may stabilize an epitope for some of the monoclonal antibodies. The other major epitopic site appears to be centered on

Tyr61 since substitution with Gly or Ala results in a molecule unable to interact with the monoclonal antibody H19. By contrast, replacement of Tyr61 with Phe or His results in a molecule that reacts normally with H19, suggesting that the presence of a bulky, phenol-containing side-chain at residue 61 is essential to maintain the H19 epitope.

Members of the Ly-6 protein superfamily to which CD59 belongs exhibit a diversity of biologic activities, all of which derive from the capacity to bind specifically to another protein. In its regulation of complement activation, CD59 interacts with both the C8 and C9 components of the C5b-9 complex to interrupt interconversion of C9 from its native conformation as found in plasma into a membrane-embedded polymer with cytolytic activity (2,3,7). CD59 can also bind CD2 expressed on T lymphocytes, and thereby mediate intracellular signaling (4-6). In each of these interactions, CD59 appears to bind selectively to a specific segment of polypeptide structure unique to each its target proteins, including complement C8 (7,37), complement C9 (7,38,39), and lymphocyte CD2 (6). In the case of the elapid snake venom toxins, the target proteins for such binding interactions include the acetylcholine receptor (for the neurotoxins) (40,41); acetylcholinesterase [fasciculin (42)], platelet integrins [mambin (43)], and a variety of other plasma membrane proteins (the cytotoxins) (20). Thus, the biologic activity of CD59, like that of the elapid snake venom toxins, is expressed through specific binding interaction with a diverse variety of other proteins. This suggests that the compact, highly-constrained disulfide-bonded structure shared among the Ly-6 superfamily of proteins represents a motif that exhibits the remarkable potential to bind to a variety of target proteins, with the specificity of each particular binding interaction imposed secondarily through the particular amino acid side chains introduced into the structure.

The present studies show that the functional binding site may not be located in homologous areas of molecules similar in structure but different in function. In erabutoxin a, an acetylcholine

receptor-binding snake toxin, residues located in the distal parts of all three loops appear to be important as mutation markedly diminishes the affinity of the toxin for the target protein (44). However, in the present studies, extensive mutation of CD59 in this area did not alter its function in inhibition of complement function. Extensive alteration of similar areas of loops 1 and 3 also failed to diminish function, suggesting that the functional site of CD59 does not reside in the peripheral extensions of the molecule.

Rushmere *et al.* (32) have suggested, based on analogies among CD59 molecules from various animals, that the functional site of CD59 is just distal to the binding site of the complex carbohydrate which, in the human molecule, is affixed to Asn18. On the other hand, our own data and data from others imply that the complement inhibitory function of CD59 does *not* critically depend on either the carbohydrate attached to Asn18 or on the amino acid side chains located in the solution structure to be near this site of glycosylation. In particular, we note that CD59 stripped of N-linked sugar retains its capacity to bind C8 and C9 normally, although some loss of function was observed when this glycanase-digested protein was added back to membranes (15). Mutagenesis of CD59 at Asn8 and Asn18 to eliminate all potential sites of N-linked glycosylation was found to have no effect on the complement-inhibitory activity of the expressed protein (45), whereas it has been reported that mutagenesis in this region of the protein is associated with an apparent increase in complement-inhibitory function (46). The present studies demonstrate that opening the disulfide bridge (Cys59-Cys64) forming a small loop located near the Asn 8 site of glycosylation also slightly increases the complement-inhibitory function of CD59. To date, no mutation in this area has been associated with a loss of this function.

Changing Tyr61 to glycine does completely abolish the function of the CD59 molecule. This could be due to either a specific change related to

the side-chain of tyrosine or the fact that substitution of glycine, which has no side chain, might allow sufficient rearrangement of the peptide backbone in the area to disrupt structure. The fact that substitution of molecules with equally bulky side-chains (phenylalanine, histidine, and alanine) does not alter function suggests either that the effect of the substitution of glycine is due to mobility and displacement of the peptide backbone or that the specific structure of tyrosine is essential for function. It is clear that the proper structure in this area of the molecule is important for its functional activity.

CD59 inhibits the lytic action of complement by preventing the formation of membrane-embedded polymers of C9 (2). Formation of this cytolytic C9 polymer is initiated through the association of plasma C9 with the C8 α subunit of the membrane C5b-8 complex. This results in a major conformational change in C9 that both exposes hydrophobic segment(s) that can intercalate with the acyl chains of membrane phospholipid as well as a binding site in C9 for the next molecule of C9, propagating the polymer. CD59 is known to interrupt this process by binding to regions of C8 α and C9 that become exposed during assembly of this membrane attack complex. The CD59 binding sites within human C8 α and C9 have recently been mapped to corresponding segments of each polypeptide that both contain a conserved intrachain disulfide-bond (Cys345-Cys369 in human C8 α and Cys359-Cys384 in human C9) (7,37-39). This segment of each complement protein is immediately C-terminal to the putative membrane-intercalating domains of C8 and C9. It can therefore be suggested that the surface of the beta-pleated central core of CD59 contained in vicinity to Tyr61 contains a complementary binding site with specific avidity for those peptide segments of human C8 α and C9 that are exposed upon their incorporation into the C5b-9 membrane attack complex. These data also suggest that the portion of CD59 that contributes to its complement-inhibitory function does not correspond to the active sites that have previously been identified in

the elapid snake venom toxin members of the Ly-6-protein superfamily, in which the binding sites are located in the exposed “loops” that radiate out from the central core of the proteins (47).

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