Role of a Disulfide-Bonded Peptide Loop within Human Complement C9 in the Species-Selectivity of Complement Inhibitor CD59†

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Received December 4, 1995⊗

ABSTRACT: CD59 antigen is a membrane glycoprotein that inhibits the activity of the C9 component of the C5b-9 membrane attack complex (MAC), thereby protecting human cells from lysis by human complement. The complement-inhibitory activity of CD59 is species-selective, and is most effective toward C9 derived from human or other primate plasma. The species-selective activity of CD59 was recently used to map the segment of human C9 that is recognized by this MAC inhibitor, using recombinant rabbit/human C9 chimeras that retain lytic function within the MAC [Hüsler, T., Lockert, D. H., Kaufman, K. M., Sodetz, J. M., & Sims, P. J. (1995) *J. Biol. Chem. 270*, 3483−3486]. These experiments suggested that the CD59 recognition domain was contained between residues 334 and 415 in human C9. By analyzing the species-selective lytic activity of recombinant C9 with chimeric substitutions internal to this segment, we now demonstrate that the site in human C9 uniquely recognized by CD59 is centered on those residues contained between C9 Cys359/Cys384, with an additional contribution by residues C-terminal to this segment. Consistent with its role as a CD59 recognition domain, CD59 specifically bound a human C9-derived peptide corresponding to residues 359−384, and antibody (Fab) raised against this C9-derived peptide inhibited the lytic activity of human MAC. Mutant human C9 in which Ala was substituted for Cys359/384 was found to express normal lytic activity and to be fully inhibited by CD59. This suggests that the intrachain Cys359/Cys384 disulfide bond within C9 is not required to maintain the conformation of this segment of C9 for interaction with CD59.

Human (hu)[1] CD59 antigen is a 18−21 kDa plasma membrane protein that functions as an inhibitor of the C5b-9 membrane attack complex (MAC) of hu complement (Davies & Lachmann, 1993). CD59 interacts with both the C8 and C9 components of MAC during its assembly at the cell surface, thereby inhibiting formation of the membrane-inserted C9 homopolymer responsible for MAC cytolytic activity (Meri et al., 1990; Rollins & Sims, 1990). This serves to protect hu blood and vascular cells from injury arising through activation of complement in plasma. CD59's inhibitory activity is dependent upon the species of origin of C8 and C9, with the greatest inhibitory activity observed when C9 is from hu or other primates. By contrast, CD59 exerts little or no inhibitory activity toward C8 or C9 of most other species, including rabbit (rb) (Okada et al., 1989; Rollins et al., 1991; Zhao et al., 1991). Because the activity of CD59 is largely restricted to regulating hu C9, and the activity of analogous complement inhibitors expressed by cells of other species is likewise generally selective for homologous C9, xenotypic cells and tissue are particularly susceptible to complement-mediated destruction due to unregulated activity of MAC. This phenomenon underlies hyperacute immune rejection after xenotransplantation (Dalmasso, 1992).

Analysis of the physical association of CD59 with components of MAC suggested that separate binding sites for CD59 are contained within the α-chain of hu C8 and within hu C9 (Ninomiya & Sims, 1992). Within C9, this site has been mapped to between residues 334 and 415 (Ninomiya & Sims, 1992; Chang et al., 1994; Hüsler et al., 1995). In this study, we examine the contribution of residues internal to this segment of hu C9 to its recognition and functional inhibition by membrane CD59.

EXPERIMENTAL PROCEDURES

Materials. Hu complement proteins C5b6, C7, C8, and C9 and hu erythrocyte membrane glycoprotein CD59 were purified and assayed as previously described (Wiedmer & Sims, 1985a,b; Rollins & Sims, 1990). Hu C9 peptide 359−384 ([allyl-K]-CLGYHLDVSLAFSEISVGAEFNKDD-[allyl]C), BSA-conjugated hu C9 peptide 359−384, and affinity-purified rb IgG against hu C9 peptide 359−384 were custom-ordered from Quality Controlled Biochemicals (Hopkinton, MA). Full-length cDNA for hu C9 was a generous gift from Dr. J. Tschopp (University of Lausanne, Epalinges, Switzerland) (Dupuis et al., 1993). Full-length cDNA for rb C9 was isolated and cloned into pSVL as previously reported (Hüsler et al., 1995). Chicken erythrocytes (chE) were from Cocalico Biologics, Inc. (Reamstown, PA), COS-7 cells were from American Tissue Culture Collection (Rockville, MD), *Escherichia coli* strain DH5α and Opti-MEM I were from Life Technologies Inc. (Gaithersburg, MD), Dulbecco's modified Eagle's medium was from Mediatech Inc. (Herndon, VA), and heat-inactivated fetal bovine serum

† This work was supported by Grant HL36061 from the Heart, Lung and Blood Institute, National Institutes of Health, and by a Research Fellowship from the American Heart Association of Wisconsin.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1996.

[1] Abbreviations: hu, human; MAC, C5b-9 membrane attack complex of complement; rb, rabbit; chE, chicken erythrocytes; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediamine tetraacetic acid; BSA, fatty acid and globulin-free bovine serum albumin.

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was from Biocell (Rancho Dominquez, CA). Oligonucleotides were synthesized by the Molecular Biology Core Laboratories, Blood Research Institute.

Solutions. MBS consists of 150 mM NaCl, 10 mM MOPS, pH 7.4; GVBS is 150 mM NaCl, 3.3 mM sodium barbital, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% (w/v) gelatin, pH 7.4; GVBE is 150 mM NaCl, 3.3 mM sodium barbital, 10 mM EDTA, and 0.1% (w/v) gelatin, pH 7.4.

Construction of Chimeric C9 cDNA's. cDNA's coding for hu/rb C9 chimeras were constructed essentially as previously described (Hüsler et al., 1995). In brief, regions of sequence identity were determined from the aligned sequences of rb and hu C9, and used as junctions for chimeric cDNA construction. Based on these alignments, primers for PCR were designed to generate defined segments of rb and hu C9 cDNA's. Primers annealing to 5′- or 3′- untranslated sequence with added *Xba*I (5′-end) or *Sac*I (3′-end) recognition sites were paired with chimeric primers (28−37 bp in length) and used to generate cDNA fragments that contained the desired overlapping sequence at either the 5′- or the 3′-ends. These fragments were gel-purified, mixed at a 1:1 molar ratio, and used in a second amplification with primers located in the 5′- and 3′-untranslated region to produce full-length chimeric C9 cDNA's. Fragments were cloned into the *Xba*I/*Sac*I sites of pSVL for mammalian expression. PCR fidelity was confirmed by sequencing the 3′-coding sequence in each construct, starting from the stop codon and continuing through all junctions of rb and hu sequence. In certain cases, chimeric constructs were further modified by site-directed mutagenesis (below).

Site-Directed Mutagenesis. C9 cDNA in pSVL served as a template for site-directed mutagenesis using the *Chameleon* mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis was performed using 0.25 pmol of template plasmid, 25 pmol of mutagenic primer, and 25 pmol of selection primer, the latter chosen to modify *Sal*I, *Sca*I, or *Xho*I restriction sites unique to pSVL. The resulting mutagenized plasmids were subject to a minimum of two rounds of selection by restriction digest, and then transformed into *E. coli* XL1-Blue (Stratagene) for single-colony isolation and plasmid purification. In all cases, mutations were confirmed by double-stranded sequencing of each purified plasmid.

Transfection of COS-7 Cells. Plasmid DNA used in transfections was obtained from purification over Qiagen-tips (Qiagen Inc., Chatsworth, CA). COS-7 cells were transfected using DEAE-dextran, and then cultured for 24 h in Dulbecco's modified Eagle's medium (Mediatech Inc.) supplemented with 10% fetal bovine serum, after which this medium was replaced by Opti-MEM I (Life Technologies Inc.). Cell supernatants were harvested after 48−65 h, PMSF (1 mM), benzamidine (1 mM), and EDTA (10 mM) were added, and the supernatants were concentrated at 4 °C (Centricon 30, Amicon).

Immunoblotting. C9 in the COS-7 supernatants was analyzed by quantitative dot blotting using murine monoclonal antibody P9-2T as previously described (Hüsler et al., 1995).

Biotin-CD59. CD59 was biotinylated by incubation (1 h, room temperature) with a 20-fold molar excess of NHS-LC-biotin in 10 mM MOPS, 0.1% Nonidet P-40, pH 9.0, followed by exhaustive dialysis against charcoal (Chang et al., 1994).

Analysis of the Inhibitory Function of CD59 toward Recombinant C9 Constructs. The hemolytic activity of each C9 construct was assayed using as target cells chE that were reconstituted with purified hu CD59 (Hüsler et al., 1995). chE were washed extensively and suspended in GVBS, and the membrane−C5b67 complex was assembled by mixing cells (1.4×10^9/mL) with C5b6 (13 μg/mL) followed by addition of C7 (1 μg/mL). After 10 min, the C5b67 chE were diluted to 1.4×10^8/mL in GVBE and incubated (10 min, 37 °C) with 0 or 750 ng/mL CD59. In each case, the final concentration of Nonidet P-40 was less than 0.002% (v/v). After washing in ice-cold GVBE, 2.8×10^6 of these cells were incubated (37 °C) in a total volume of 100 μL with 1 ng of rb C8 plus 0−50 ng of recombinant C9, serially-diluted in Opti-MEM I. Hemolysis was determined after 30 min at 37 °C, with correction for nonspecific lysis, determined in the absence of C9. In each experiment, the inhibitory activity of CD59 toward each recombinant C9 construct was determined from the reduction in complement lysis of those cells reconstituted with CD59, versus the identically-treated cells omitting CD59, measured at the midpoint of the C9 titration (i.e., 50% hemolysis) (Hüsler et al., 1995). In order to directly compare results obtained in experiments performed on different days, data for each recombinant C9 construct were normalized to results obtained in each experiment with hu C9.

CD59 Binding to Hu C9 Peptide 359−384. The specific binding of CD59 to hu C9-derived peptide 359−384 was measured by microtiter plate assay with biotin-CD59, according to modification of published methods (Chang et al., 1994; Hüsler et al., 1995). Briefly, the BSA−peptide conjugate was adsorbed to 96-well polyvinyl microplates by overnight coating at 5 μg/mL in 0.1 M sodium bicarbonate, pH 8.5. After blocking with 1% (w/v) BSA, wells were washed and incubated (4 h, 37 °C) with 0.5−1 μg/mL biotin-CD59. After the wells were washed, the bound biotin-CD59 was detected with Vectastain (Vector Labs, Burlingame, CA), developed by addition of *p*-nitrophenyl phosphate (2 mg/mL), and the optical density was recorded at 405 nm (VMaxMicroplate Reader, Molecular Devices, Inc.). In all experiments, correction was made for the background adsorption of biotin-CD59 to BSA-coated wells (no peptide) and for nonspecific binding of biotin-CD59 to peptide, determined in the presence of a 20-fold excess of unlabeled CD59. As a positive control for specific binding, comparison was made in each experiment to wells coated with 2 μg/mL hu C9 (Chang et al., 1994). The capacity of monospecific antibody against hu C9 peptide 359−384 to compete specific binding of CD59 was determined by prior incubation of the BSA−peptide-coated wells with antibody (2 h, 0−100 μg/mL IgG) before addition of biotin-CD59.

Inhibition of MAC Lysis by Antibody against Hu C9 Peptide 359−384. The capacity of antibody against hu C9 peptide 359−384 to inhibit MAC was determined by hemolytic assay, using the chE target cells described above, omitting CD59. In these experiments, 0−1 mg/mL Fab of antibody against hu C9 peptide 359−384 (or nonimmune antibody control) was added with recombinant C9 (hu, rb, or chimeric), and complement-specific lysis was determined.

RESULTS

C9 chimeras were constructed in which the segments of C9 corresponding to the putative CD59 binding site [residues

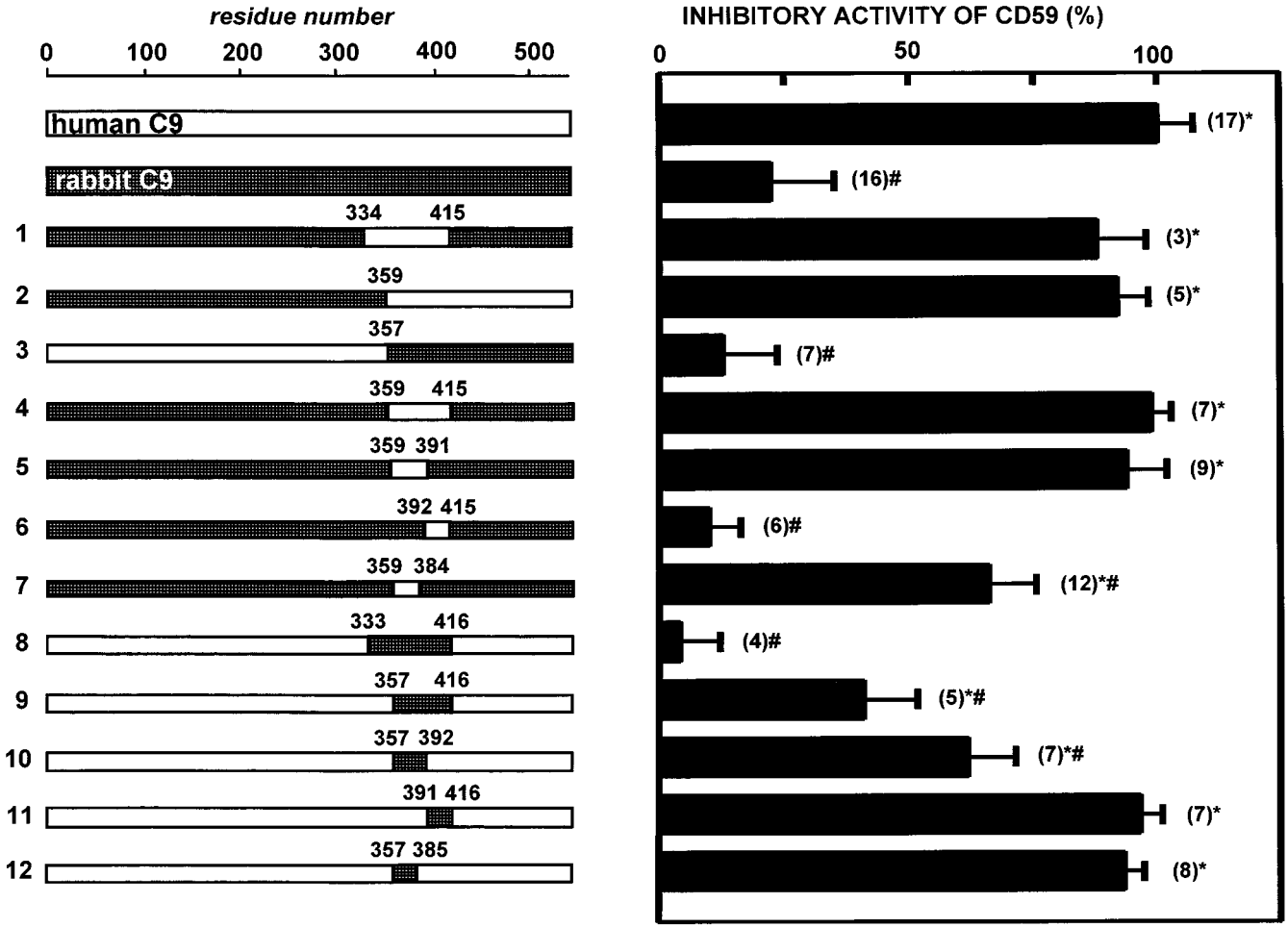

FIGURE 1: Inhibitory activity of CD59 toward hu/rb chimeras of complement C9. Bar graph (right panel) summarizes combined results of all experiments measuring the inhibitory activity of CD59 toward recombinant hu/rb chimeras of C9. In each assay, hemolytic titrations of C9 were performed against C5b-8 chE in the presence and absence of membrane CD59, and the percent reduction of hemolysis due to CD59 (ordinate) was determined, with normalization to that observed for hu C9 (100% inhibition) as described under Experimental Procedures. Error bars denote mean ± SD, parentheses indicate number of independent experiments; asterisks (*) indicate significance ($p < 0.01$) when compared to rb C9; pound signs (#) indicate significance ($p < 0.01$) when compared to hu C9. To the left of each data bar, the protein assayed is depicted so as to designate those portions of the polypeptide containing hu C9 (open) or rb C9 (shaded) sequence. Numbers above each construct indicate the junctional hu C9 residue at each transition between hu and rb protein sequence. Bars designated as human C9 and rabbit C9 denote recombinantly-expressed hu and rb C9, respectively. Recombinant C9 chimeras (designated as 1−12) contain human (H) or rabbit (R) sequence according to the deduced mature primary structure of hu and rb C9. In some C9 chimeras, the numbering appears discontinuous because of gaps in the alignment of the hu and rb sequences: 1, R1−338H334−415R425−536; 2, R1−363H359−538; 3, H1−357R363−536; 4, R1−363H359−415R425−536; 5, R1−363H359−391R401−536; 6, R1−400H392−415R425−536; 7, R1−363H359−384R394−536; 8, H1−333R339−424H416−538; 9, H1−357R363−424H416−538; 10, H1−357R363−400H392−538; 11, H1−391R401−424H416−538; 12, H1−357R363−393H385−538.

334−415 in hu C9 (Chang et al., 1994; Hüsler et al., 1995)] were interchanged between hu and rb C9. These chimeric proteins were then tested for hemolytic activity and for their sensitivity to inhibition by membrane CD59 (Figure 1). Substitution of hu C9 residues 334−415 into rb C9 (chimera 1) resulted in a protein that was indistinguishable from hu C9 in its sensitivity to inhibition by CD59. Conversely, when this same segment of hu C9 was replaced by the corresponding rb C9 sequence (chimera 8), the resulting chimera was indistinguishable from rb C9 and virtually unaffected by the presence of membrane CD59. In these experiments, MAC was assembled using hu C5b67 and rb C8 so as to circumvent the known inhibitory interaction of CD59 with hu C8 (Rollins et al., 1991; Ninomiya & Sims, 1992).

As depicted in Figure 2, the segment of hu C9 shown to bind CD59 is immediately C-terminal to the putative membrane-spanning domain of the protein, and corresponds to a segment of polypeptide exhibiting particularly low sequence conservation when hu C9 is aligned to C9 of rb or other nonprimate species (Hüsler et al., 1995). The most prominent divergence of sequence occurs between two cysteines (Cys359−Cys384 in hu C9) that are conserved in the hu and rb proteins. In hu C9, these cysteines have been shown to form an intrachain disulfide bond (below) (Schaller et al., 1994).

In order to further localize the segment of hu C9 recognized by CD59 and to determine the specific contribution of residues spanning the Cys359/384 disulfide, we constructed a series of hu/rb C9 chimeras by interchanging segments of corresponding hu and rb C9 sequences internal to residues 334−415. Each of these chimeric proteins was expressed and analyzed for MAC hemolytic function, and for sensitivity to inhibition by membrane CD59. All resulting hu/rb C9 chimeras were functionally active as determined by hemolytic titration against chE containing membrane C5b-8 (*not shown*). Analysis of CD59-inhibitory activity toward each of these proteins revealed (Figure 1) the following: (i) Inhibition of MAC lytic activity by CD59

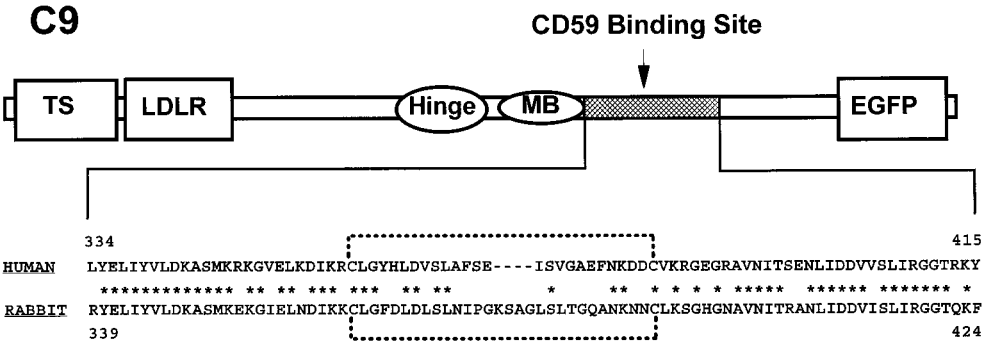

FIGURE 2: Segment of hu C9 identified as containing the CD59 binding site. Schematic representation of hu C9 according to the proposed domain structure which includes thrombospondin type 1 (TS), LDL receptor (LDLR), hinge (Hinge), membrane binding (MB), and epidermal growth factor precursor (EGFP) domains. The shaded segment indicates residues 334−415 of hu C9, spanning the putative CD59 binding site [from Hüsler et al. (1995)]. The amino acid sequence of this peptide segment is given below, and is shown in an alignment with rb C9 (alignment done for full-length polypeptides with the PALIGN program in PCGENE). Asterisks indicate sequence identity. Dotted lines indicate the Cys 359/384 disulfide of hu C9 and the assumed corresponding internal disulfide in rb C9. Residue numbers refer to the mature proteins.

was unaffected by replacement of all residues N-terminal to Cys359 of hu C9 with the corresponding rb sequence (chimera 2), whereas replacement of all residues C-terminal to residue 357 of hu C9 with the corresponding rb sequence (chimera 3) resulted in a protein indistinguishable from rb C9 and only weakly inhibited by CD59. Consistent with the results for chimeras 1−3, substitution of hu C9 residues 359−415 into the corresponding segment of otherwise rb C9 (chimera 4) resulted in a protein that was indistinguishable from hu C9, suggesting that this polypeptide segment of hu C9 (residues 359−415) contains the binding site for CD59. (ii) To further resolve the segment of hu C9 required for species-selective interaction with CD59, additional chimeras were constructed further truncating the segment of hu sequence substituted into rb C9 (chimeras 5−7). Data for these chimeras revealed that whereas hu residues 359−391 conferred full recognition by CD59 (chimera 5), hu C9 residues 392−415 failed to confer any recognition by CD59 when inserted into an otherwise rb C9 (chimera 6). Truncation of the inserted segment of hu C9 sequence from 359−391 (chimera 5) to 359−384 (chimera 7) was accompanied by a small but significant reduction in inhibition of MAC lytic activity by CD59. These results imply that CD59 directly interacts with the segment of hu C9 contained between residues 359 and 391, with the peptide segment spanning the intrachain Cys359/384 disulfide substantially contributing to this interaction. (iii) We next attempted to abrogate CD59's interaction with hu C9 by replacement of sequence spanning this putative CD59 recognition domain with corresponding rb sequence (chimeras 8−12). Replacement of hu C9 residues 334−415 with corresponding rb sequence (chimera 8) completely eliminated hu-selective interaction with CD59, as anticipated for results obtained for the complementary construct, chimera 1. Nevertheless, when the segment of rb-derived sequence substituted into otherwise hu C9 was further truncated, the resulting chimeras (chimeras 9−12) retained a surprising degree of sensitivity to the inhibitory effects of CD59, characteristic of hu C9. Thus, substitution of rb sequence for the residues internal to Cys359−384 of hu C9 (chimera 12) did not significantly diminish CD59's capacity to inhibit the lytic activity of C9, while C-terminal extension of the segment of rb sequence to residue 415 (chimera 9) did not completely eliminate interaction with CD59. Taken together with results for chimeras 1−5, these data imply that whereas hu C9 residues

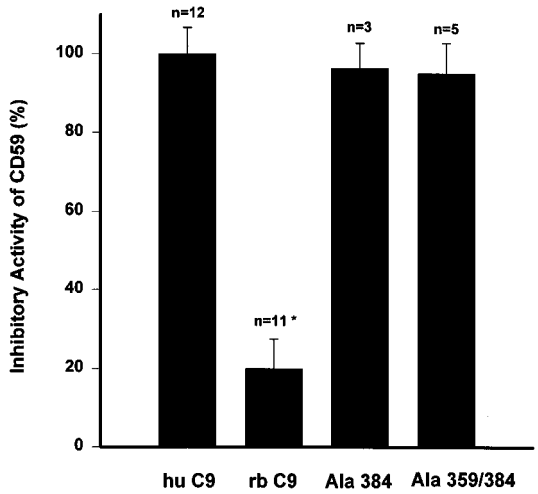

FIGURE 3: Inhibitory activity of CD59 is unaffected by disruption of the Cys 359/384 disulfide. Recombinant hu C9 was expressed with a Cys → Ala mutation either at residue 384 or at both residues 359/384, and analyzed as described in Figure 1. Inhibitory activity of CD59 toward the hemolytic function of each recombinant C9 is expressed as a percentage, relative to that measured for wild-type hu C9 (ordinate). Error bars denote mean ± SD; *n* indicates number of independent experiments; asterisks indicate significance ($p < 0.001$) compared to hu C9. Hu C9 and rb C9 denote the wild-type hu and rb proteins, respectively.

359−391 alone are sufficient to confer recognition by CD59, segments of the polypeptide immediately flanking this segment significantly contribute to the extent to which this binding site is expressed (see Discussion).

The Cys359/384 disulfide in hu C9 has recently been reported to be highly labile and subject to spontaneous reduction in the native protein (Hatanaka et al., 1994). Since our data suggested that residues internal to Cys359/384 contribute in-large-part to species-selective recognition by CD59, we examined the extent to which the CD59 recognition site in C9 is affected by disruption of this bond. Mutant hu C9 was expressed with Ala substitutions at Cys359 and Cys384 and tested for hemolytic activity and for sensitivity to inhibition by CD59. As revealed by the data of Figure 3, disruption of this disulfide bond did not significantly affect the hemolytic activity of the protein nor the capacity of CD59 to specifically inhibit C9 lytic activity. This suggests either that the segment of hu C9 forming the CD59 binding site is either conformationally constrained independent of the Cys359−384 disulfide or that this binding site is expressed

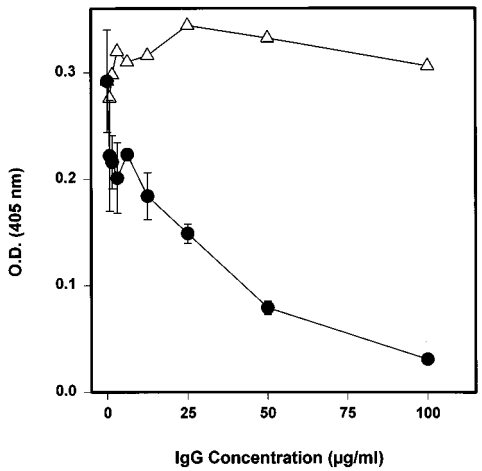

FIGURE 4: CD59 specifically binds hu C9 peptide 359−384. Microplates were coated with hu C9 peptide 359−384 coupled to BSA, and specific binding of biotin-CD59 was determined in the presence of affinity-purified antibody against hu C9 residues 359−384 (●), or nonimmune IgG (△) (IgG concentrations indicated on abscissa). All data were corrected for nonspecific binding of CD59, determined in the presence of a 20-fold excess of unlabeled CD59 (see Experimental Procedures). Ordinate denotes absorbance at 405 nm, with correction for nonspecific background. Error bars denote mean ± SD. Data of a single experiment, representative of three so performed.

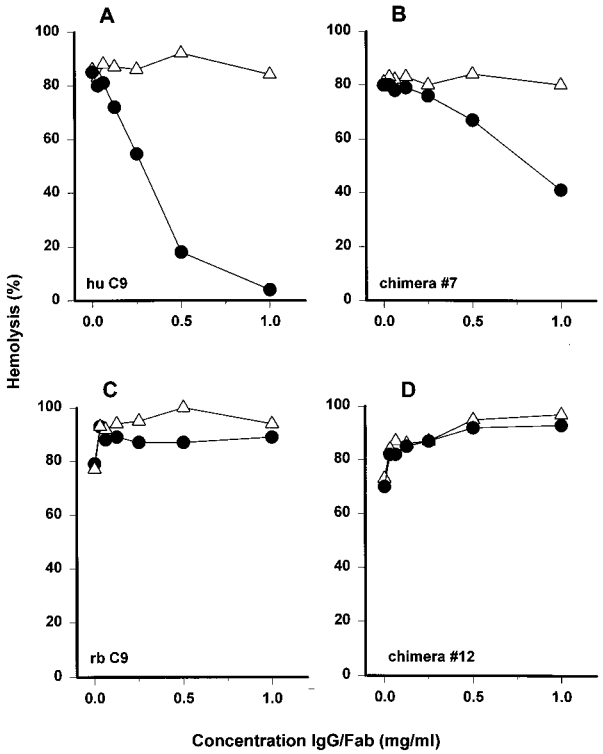

FIGURE 5: Inhibition of C9-dependent lysis by antibody against C9 peptide 359−384. Fab of antibody against hu C9 peptide 359−384 (●) was tested for its capacity to inhibit the hemolytic activity of recombinant hu C9 (panel A), hu/rb C9 chimera 7 (panel B), recombinant rb C9 (panel C), or hu/rb C9 chimera 12 (panel D). Residues of human (H) and rabbit (R) sequence in each C9 chimera are indicated in Figure 1. Also shown are data for nonimmune antibody (△) (final concentrations indicated on abscissa). In all experiments, C5b-8 chE lacking CD59 served as target cells, and hemolysis was measured with correction for nonspecific lysis as described under Experimental Procedures. Data of single experiment, representative of three similar experiments.

in the primary structure of hu C9, independent of protein folding. In this regard, it is of interest to note that we have previously observed specific binding of labeled CD59 to reduced and carboxymethylated hu C9, as well as to linear peptides that contain the hu C9-derived sequence that we now identify as the CD59 recognition domain of the polypeptide (Ninomiya & Sims, 1992; Chang et al., 1994; Hüsler et al., 1995).

In order to confirm that the peptide segment spanning hu C9 359−384 can itself mediate interaction with CD59, this 26 residue peptide was synthesized, coupled to BSA, and analyzed for CD59 binding, using biotin-CD59 conjugate in a microplate assay. As demonstrated by Figure 4, biotin-CD59 specifically bound to C9 peptide 359−384, and this binding was inhibited by excess unlabeled CD59 or by antibody directed against the peptide.

CD59 is known to bind to C9 after C9 incorporates into the C5b-9 complex, and through this interaction inhibit propagation of membrane-inserted C9 polymer, limiting the lytic activity of MAC. In order to confirm the importance of the peptide segment recognized by CD59 to MAC assembly, Fab of antibody raised against the hu C9 peptide 359−384 was tested for its capacity to inhibit the hemolytic activity of the hu C5b-9 complex, under the same conditions used to evaluate the inhibitory function of CD59. As shown by the data of Figure 5, this Fab inhibited the hemolytic activity of hu C9 and C9 chimera 7 (representing rb C9 containing hu C9 residues 359−384; Figure 1), but had no effect on the hemolytic activity of either rb C9 or chimera 12 (representing substitution of the corresponding segment of rb C9 residues into hu C9; Figure 1).

DISCUSSION

Our experiments suggest that hu C9 residues 359−384 promote CD59 binding, and that this segment of hu C9 contributes to the species-selective regulation of MAC function, providing an initial clue to the structural motif(s) through which this inhibitor selectively regulates the lytic activity of the hu C5b-9 complex. These data further suggest that the capacity of CD59 to optimally interact with this segment of hu C9 is significantly influenced by residues immediately C-terminal to this segment of the C9 polypeptide.

Whereas our data establish that residues internal to Cys359−Cys384 contribute to recognition by CD59, the disulfide bond between these two Cys is apparently not required either for maintenance of C9's hemolytic activity within MAC or for normal regulation of that activity by membrane CD59. These conclusions derived by Cys/Ala mutagenesis in recombinant hu C9 (Figure 3) are consistent with previous reports indicating: (i) the intrinsic lability of the Cys359−384 disulfide in C9 purified from hu plasma, where spontaneous reduction of this bond did not appear to alter C9 hemolytic activity (Hatanaka et al., 1994); and (ii) that a specific CD59 binding site is retained in reduced and carboxymethylated hu C9, in hu C9-derived peptide fragments, and can be demonstrated for *E. coli* fusion proteins containing hu C9-derived sequence spanning residues 359−384 (Ninomiya & Sims, 1992; Chang et al., 1994). This suggests that the CD59 binding site expressed by this segment of hu C9 reflects interactions between amino acid side chains that do not require formation of the Cys359/Cys384 disulfide bond.

As noted above, chimeras generated by substituting limited segments of hu C9 into rb C9 revealed that the segment of hu C9 between 359 and 384 uniquely conferred recognition by CD59, and that this interaction was enhanced by C-terminal extension of the hu sequence to residue 391 (cf. chimeras 1−7; Figure 1). Surprisingly, chimeras generated by replacing these same segments of hu C9 with corresponding rb C9 sequence did not exhibit a complementary decrease in the interaction with CD59, except when the segment of rb-derived sequence replaced all hu C9 residues spanning 334−415 (cf. chimeras 8−12; Figure 1). A possible explanation for this apparent discrepancy is that residues within the 359−391 segment of hu C9 that contribute to the CD59 binding site are partially conserved in the corresponding segment of rb C9, but that the high-avidity interaction uniquely observed for hu C9 reflects a conformation of the polypeptide that is intrinsic only to the hu, but not rb, C9 sequence. Whereas this structural motif appears to be inherently favored by hu residues 359−391 irrespective of the species of origin of flanking C9 sequence (e.g., chimera 5), in the case of the corresponding segment of rb C9 sequence, appropriate folding to express a high-avidity CD59 binding does not normally occur, but may be partially induced by imposition of appropriate flanking hu C9 sequence.

In addition to interacting with the C9 component of hu MAC, CD59 is also known to interact with hu C8α, a polypeptide that exhibits sequence similarity to hu C9 and to several other MAC components (Ninomiya & Sims, 1992; Lockert et al., 1995). The CD59 recognition domain in hu C8α as deduced from analysis of the lytic activity of hu/rb C8α chimeras and peptide binding was localized to residues 320−415 (Lockert et al., 1995). It is of interest to note that from the aligned sequences of hu C8α and hu C9, the peptide segment in C9 that we now identify as conferring recognition by CD59 (C9 359−391) overlaps the same region of C8α that was shown to contain the CD59 recognition domain. The aligned amino acid sequences of hu C8α and C9 that span the respective CD59 recognition sites of these two proteins reveal very limited sequence identity and unremarkable sequence homology. This suggests either (1) that these two polypeptides each fold so as to align the conserved amino acid side chains within their respective CD59 binding domains into a common motif that is recognized by CD59 or (2) that the CD59 binding sites expressed by C8α and C9 are not structurally identical, but are comprised of distinct motifs that are individually recognized by the inhibitor. In this context, it is of interest to note that whereas the CD59 binding site in hu C9 does not require formation of the intrachain disulfide between C9 Cys359/384, CD59 binding to C8α depends upon the integrity of the corresponding internal disulfide (C8α Cys 345/369) as well as allosteric effects of the flanking C8α sequence and the sequence contained in the hu C8β chain (Lockert et al., 1995).

Neither the tertiary structure nor the biologic function of the segment of hu C9 containing the CD59 recognition site is known. It is of note that the CD59 binding site is not constitutively expressed by native C9 as found in hu plasma, and is only expressed once C9 binds to C5b-8. Alternatively, the CD59 binding site in C9 can be exposed through partial denaturation, as achieved by surface adsorption or through transfer to nitrocellulose (Rollins et al., 1991; Ninomiya & Sims, 1992; Chang et al., 1994; Hüsler et al., 1995). This implies that the binding site recognized by CD59 is normally buried in the native solution structure of C9, becoming exposed through the conformational rearrangement that is induced upon C9's incorporation into MAC. Our data indicate that through direct binding to this site, CD59 or Fab fragments specific for the hu C9 359−384 peptide sequence interrupt MAC assembly, inhibiting C5b-9 lytic function. This raises the possibility that this segment of C9 is directly involved either in C9's insertion into the target membrane or in C9−C9 interaction required for propagation of C9 polymer. Alternatively, this segment may represent a pivot point in C9 through which polypeptide chain rearrangement to expose these functionally-important segments occurs. The latter possibility would seem most likely based on the following considerations: As discussed above, whereas C9 from different species function interchangeably within MAC, only primate C9 exhibits marked sensitivity to inhibition by CD59, consistent with the highly-divergent sequence noted for the segment of hu C9 to which CD59 binds, when aligned with C9 from other species. This suggests that the CD59 binding site in hu C9 is unlikely to also provide the highly-conserved functions of C8/C9 binding and membrane insertion. Insight into how CD59's interaction with that portion of C9 contained between residues 359 and 391 confers its MAC inhibitory function awaits further information as to how this region participates in the conformational transitions required to convert plasma C9 into a membrane-inserted protein. Interestingly, the segment recognized by CD59 is in close proximity within the primary structure to the proposed membrane-interacting domain in C9 (residues 292−333; see Figure 2) (Peitsch et al., 1990). Whereas this domain is presumed buried in the conformer of C9 found in plasma, binding to membrane C5b-8 is thought to initiate unfolding so as to expose this domain for direct interaction with membrane lipid (Stanley, 1989; Peitsch et al., 1990). By binding near this site, CD59 might prevent its exposure and thereby interrupt C9 insertion into the membrane.

ACKNOWLEDGMENT

The technical assistance of Diana Schick, Lilin Li, and Robert Luhm is gratefully acknowledged.

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BI952862W