Elimination of Potential Sites of Glycosylation Fails to Abrogate Complement Regulatory Function of Cell Surface CD59*

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CD59 is a glycosylphosphatidylinositol-anchored membrane glycoprotein that serves as the principle cellular inhibitor of the C5b-9 membrane attack complex (MAC) of human complement. Approximately 50% of the total apparent mass of CD59 is attributable to glycosylation of a single Asn (Asn18). The deduced amino acid sequences of CD59 homologues identified in Old and New World primates as well as in rat reveal that the motif for N-linked glycosylation at the residue corresponding to Asn18 of human CD59 is invariably conserved, despite considerable sequence divergence elsewhere in the protein. Such conservation suggests that the post-translational modification at Asn18 has importance for either expression or normal function of CD59 at the cell surface. In this study, we specifically examined how deletion or transposition of the site of N-linked glycosylation in the CD59 polypeptide affects its MAC inhibitory function. Our data demonstrate that the inhibitory potency of CD59 is unaffected when glycosylation is transposed from Asn18 to another site in the polypeptide. Furthermore, we show that CD59 retains normal MAC regulatory function when mutated to eliminate all potential sites for N-linked glycosylation. These data suggest that the MAC inhibitory function of CD59 is entirely provided by residues exposed at the surface of the core polypeptide and that this core structure is not influenced by glycosylation at Asn18.

CD59 is a human glycosylphosphatidylinositol-anchored membrane glycoprotein that serves as the principle inhibitor of the cytolytic and pore forming activities of the C5b-9 membrane attack complex (1). By inhibiting MAC, CD59 protects human blood cells, vascular endothelium and other cells from complement, which is normally present in human plasma and other body fluids. Although the exact mechanism of its complement inhibitory function remains to be elucidated, CD59 is known to bind peptide segments of C8 and C9 that become exposed when these complement proteins incorporate into the C5b-9 complex, thereby preventing initiation or propagation of the membrane-inserted C9 polymer that is responsible for membrane damage (2, 3). The activity of CD59 exhibits prominent species selectivity, with the greatest inhibition observed when MAC is assembled from C8 and C9 of human or other primate origin (4–6).

Based upon sequence and solution structure, CD59 belongs to the snake venom/LY6 protein superfamily, whose members include cardiotoxins, erabutoxins, and the murine lymphocyte Ly6 antigen (7–9). These proteins contain a characteristic disulfide-bonded polypeptide core comprised of a four-stranded β-sheet apposed to a two-strand β-finger. In addition to the C-terminal glycosylphosphatidylinositol anchor at residue 77, a prominent feature of the post-translational modification of CD59 is the addition of a highly branched carbohydrate moiety to residue Asn18, which accounts for approximately 30–50% of the total apparent mass of CD59 (10). The solution structure of CD59 suggests that this carbohydrate occludes one face of the disc-like structure formed by the core polypeptide. In human erythrocyte CD59, a second potential motif for N-linked glycosylation (Asn5) is not utilized.

The deduced amino acid sequences of CD59 homologues identified in Old and New World primates as well as in rat reveal that the motif for N-linked glycosylation at the residue corresponding to Asn18 of human CD59 is invariably conserved, despite considerable sequence divergence elsewhere in the protein (11–14). Such conservation suggests that the post-translational modification at Asn18 may have importance for either expression or normal function of the protein at the cell surface. Recently, a viral CD59 homologue was identified in the genome of Herpesvirus saimiri (HVS) that lacks the N-linked glycosylation site at position 18 but contains a new potential locus of N-linked glycosylation at Asn5 (15). This change in the presumptive site of N-linked glycosylation in HVS CD59 reflects two separate mutations in the CD59 homologue gene of the squirrel monkey, the natural host of HVS from which the HVS CD59 gene was apparently acquired (12). Despite the absence of the conserved Asn18 glycosylation site, increased resistance to cell lysis by MAC was detected when HVS CD59 was expressed on the surface of eukaryotic cells (12). This suggests that glycosylation of CD59 at Asn18 is not required for normal surface expression and function or that these function(s) are provided when N-linked glycosylation occurs elsewhere in the polypeptide. In this study, we specifically examine how deletion or transposition of the site of N-linked glycosylation in the CD59 polypeptide affects its MAC inhibitory function. Our data demonstrate that the inhibitory potency of CD59 is unaffected when glycosylation is transposed from Asn18 to Asn5 and that the core polypeptide retains normal MAC regulatory function even when mutated to eliminate all potential sites for N-linked glycosylation.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strain Top 10 and pcDNA3 vector were from Invitrogen Corp. (San Diego, CA). All restriction endonucleases

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1 The abbreviations used are: MAC, C5b-9 membrane attack complex of complement; HVS, Herpesvirus saimiri; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; HBSS, Hanks’ balanced salt solution; BCECF-AM, 2’,7’-bis(2-carboxyethyl)-5-(and 6-carboxy-fluorescein acetoxyethyl ester; PCR, polymerase chain reaction; mAb, monoclonal antibody; MOPS, 4-morpholinepropanesulfonic acid.
Mutational Analysis of Glycosylation Site in CD59

were from New England Biolab (Beverly, MA). T4 DNA ligase, Hanks' balanced salt solution (HBSS), and DTT were from Life Technologies, Inc. Wizard kit was from Promega (Madison, WI). T4 polymerase was from Perkin-Elmer. Sequenase version 2.0 kit was from U.S. Biochemical Corp. SV-T2 cell line (ATCC 163.1) was from American Type Culture Collection (Rockville, MD). Fetal bovine serum, calf serum, bovine serum albumin (BSA), cell dissociation solution, and other common chemicals were from Sigma. BCEF-CAM-AM was from Molecular Probes (Eugene, OR). N-Glycanase was from Genzyme (Cambridge, MA). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was from Jackson Immunoresearch Laboratory (West Grove, PA). Rabbit anti-mouse lymphocyte IgG was the product of Inter-cell Technologies (La Jolla, CA). Plasmid pCDNA3, generated previously (12) using a 5'-PstI sequence coding for the mutated mature protein and 3'-untranslated sequence and approximately 115 base pairs containing 5'-CCGTCCAATGTTCA-3' from unbound antibody by centrifugation (10 min, 12,000 g) over 1 ml of oil mixture containing 5% dithiobital phthalate and 15% Apiezon (Apiezon Products, LTD, London, UK). The supernatant and oil layer were aspirated, and the radioactivity of the cell pellet was measured by photon counting with correction for nonspecific binding to mock pCDNA3-transfected SV-T2 cells.

Plasmid Construction—Glycosylation sites of human CD59 were generated through PCR mutagenesis. To generate a nonglycosylated point mutations (5'-GGTCATAGCCTGCAGTGCTACAACTGTCCTCA-3') into the 5'-end of the mature molecule that contained the appropriate and a 3' primer homologous to SP6 sequence found in the vector (5'-'C-ATACGATTGGTGGACATAG-3'). Briefly, 10 ng of plasmid DNA were used as a template in a 100-μl PCR reaction (50 mM KCl, 10 mM Tris HCl, pH 9.0, 1.5 mM MgCl₂, 0.1% gelatin (w/v), 1.0% Triton X-100, 200 μM each dNTP, 2.5 units of Taq DNA polymerase, Perkin-Elmer) using 25 pmol of each primer. Thermalcycler conditions were as follows: 95°C, 1 min; 55°C, 1 min; 72°C, 1 min; 20 cycles; 72°C 10 min. The PCR fragment was cloned into the pCR II vector of the TA cloning kit using the manufacturer's protocol (Invitrogen, San Diego, CA). DNA sequence was verified using the dye-charge chain termination sequencing method. The mutated CD59 cDNA was subcloned into pCDNA3 using a three-way ligation of the following DNA fragments: a fragment of approximately 115 base pairs containing 5'-untranslated sequence and leader sequence excised from pCDNA3/CDS9 using endogenous BamHI and PstI sites; a fragment of approximately 312 base pairs containing sequence coding for the mature molecule and 3'-untranslated sequence excised with PstI from the pCR II vector; and the pCDNA3 expression cassette digested with BamHI/EcoRI. PCR amplification and subcloning of CD59 containing a unique N-linked glycosylation motif (Asn5'Gln/Asn21'Gln) that mimics that of HVS CD59 was generated through PCR mutagenesis. To generate a nonglycosylated motif for glycosylation at a corresponding site (Asn16 of rat CD59; Asn21 of squirrel monkey CD59) is conserved in all mature protein were eliminated by substituting glutamine for aspartate at amino acid position seven (5'-GGTCATAGCCTGCAGTGCTACAACTGTCCTCA-3').

Expression in SV-T2 Cells—SV-T2 cells were transfected with CD59-pCDNA3 and mutant variants by the calcium phosphate-BBS method (18, 19). In brief, 10⁵ SV-T2 cells with 9 ml of DMEM complete medium (10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 0.4 mg/ml streptomycin, and 0.02 mM fungizone) in 6-well plates were mixed with 1 ml of transfection mixture containing 30 μg of plasmid DNA, 0.125 mM CaCl₂, 1 mM BBS, pH 6.96, and incubated at 3% CO₂, 35°C for 24 h. Transfected cells were washed twice with HBSS and replaced with 3 ml of DMEM complete medium and incubated at 37°C, 5% CO₂. After 48 h, stable transfecteds were selected for 8 days with DMEM complete medium containing 1 mg/ml geneticin. The genein-sclected cells were stained with mAb 10G10 followed with fluorescein isothiocyanate-conjugated goat antibody against mouse IgG, sorted by FACStar (Becton Dickinson) and then cloned by limited dilution. Comparison was made to clonal cell lines derived by transfection with CD59-pCDNA3 and to pCDNA3 (lacking insert).

Fluorescence Measurement of Antigens—Transfected SV-T2 cells grown to near-confluence were detached with cell dissociation solution and adjusted to 10⁷ cells/tube. The cells were incubated 30 min at 23°C with mAb 10G10 (5 μg/ml) in 30 μl of HBSS containing 1% BSA. After washing with HBSS, the cells were incubated (20 min, 23°C) with fluorescein isothiocyanate goat anti-mouse IgG at a final concentration of 5 μg/ml, and fluorescence was determined by flow cytometry (FACScan; Becton Dickinson).

Affinity Purification of Antibodies—The IgG fraction of mAb 10G10 was labeled with [125I]iodine using Iodogen, (20) and protein was separated from free iodote by Sephadex G25 (PD-10, Pharmacia Biotech Inc.) followed by exhaustive dialysis. The specific radioactivity was 2800 cpm/ng of antibody.

Quantitation of Cell Surface CD59—Expression of recombinant CD59 on the surface of transfected SV-T2 cells was determined by the binding of anti-CD59 mAb 10G10-115I/10G10 measured at saturation. The subconfluent SV-T2 cell monolayer was suspended with cell dissociation solution and incubated for 3 min with 1% paraformaldehyde. After fixation, the cells were collected, washed twice, and suspended in HBSS-1% BSA containing 50 μg/ml 115I-10G10. After 1 h at 37°C, 3 x 10⁶ cells were diluted in 0.5 ml of Tris-buffered saline and separated from unbound antibody by centrifugation (10 min, 12,000 x g) over 1 ml of oil mixture containing 5% dithiobital phthalate and 15% Apiezon (Apiezon Products, LTD, London, UK). The supernatant and oil layer were aspirated, and the radioactivity of the cell pellet was measured by photon counting with correction for nonspecific binding to mock pCDNA3-transfected SV-T2 cells.

Immunoaffinity Purification of CD59—CD59 was purified from transfected SV-T2 cells expressing 0.6-2.5 x 10⁹ CD59 molecules/cell by immunoaffinity absorption on rat monoclonal antibody YTH53.1 against CD59. Membrane proteins were extracted with 2% Triton X-100 in Dulbecco's phosphate-buffered saline containing 10 mM EDTA, 50 mM Tris HCl, 100 mM NaCl, 5% prorline at amino acid position seven (5'-GGTCATAGCCTGCAGTGCTACAACTGTCCTCA-3').

Western Blotting—Recombinant CD59 (wild type or mutant) from detergent extracts of transfected SV-T2 cells was electrophoresed in a 8–25% gradient gel and transferred to a nitrocellulose membrane (PhastSystem, Pharmacia). After blocking for 1 h with 1% BSA-Triton-buffered saline, the washed nitrocellulose membrane was incubated for 1 h with mAb 10G10 (1 μg/ml) in 1% BSA-Triton-buffered saline, washed, and incubated 1 h with a 1:8000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG. The washed blots were developed with chemiluminessence substrate (DuPont NEN), and bands were visualized by brief exposure to x-ray film. All procedures were performed at 23°C.

C9b-9 Inhibitory Activity Assay—The complement inhibitory activity of recombinant CD59 in SV-T2 was evaluated by the measurement of resistance of these cells to C9b-9-mediated efflux of trapped dye (BCEF-CAM) from the cytoplasm as described previously (6). SV-T2 cells grown to 80% confluence in 48-well cell culture plate were washed twice with 1% BSA-HBSS, incubated with 15 μg BCECF-CAM, and 1.5 mg/ml rabbit anti-mouse lymphocyte IgG for 15 min at 37°C. After washing, human serum depleted of complement protein C9 (25%) and human serum (0.8 μg/ml) were added. Cell lysis was determined by the release of BCECF fluorescence into the supernatant. Total cell-associated dye was determined from a 10% SDS-treated cell lysate. The C9b-9-mediated dye release was expressed as a percentage relative to total, with correction for nonspecific dye release and background fluorescence measured for identically treated controls omitting C9. In all cases, BCECF fluorescence was determined by transfer of 100-μl aliquots of the cell-free supernatants or detergent lysates into 96-well plate and measured with CytoFluor II Fluorometer. The Microtiter Reader with excitation at 485 nm and emission at 530 nm.
mammalian CD59 homologues sequenced to date (11-14). To determine whether this highly conserved site of N-linked glycosylation is required either for normal expression or complement regulatory function of the protein, two glycosylation variants of CD59 were generated through PCR mutagenesis (Fig. 1). One variant (Gln8/Gln18) was generated by converting the asparagine residues at amino acid positions 8 and 18 to glutamine residues, resulting in a molecule that lacks any N-linked glycosylation motifs. The other variant, Asn5/Gln8/Gln18, contained the same mutations described for Gln8/Gln18 with the addition of a proline to serine change at amino acid position 7. This change introduced a unique glycosylation motif into CD59 that mimics the putative glycosylation site identified in HVS CD59. As illustrated by Fig. 2, Western blot analysis of each recombinant protein before and after N-glycanase treatment confirmed the presence of N-linked sugar in both wild type CD59 and the Asn5/Gln8/Gln18 mutant, with the glycosylated protein exhibiting an apparent molecular mass of 21 kDa. No such N-linked sugar could be detected in the Gln8/Gln18 mutant (apparent molecular mass, 14 kDa), consistent with the elimination of all potential sites of N-glycosylation in this construct. By contrast to human erythrocyte-derived CD59, which was always completely glycosylated (21 kDa), the extent of glycosylation of recombinant CD59 (wild type and N5 mutant) extracted from the transfected SV-T2 cells was found to vary, presumably reflecting the residual intracellular pool of newly synthesized protein (14 kDa) that has not processed through the Golgi.

**Cell Surface Expression of Wild Type and Mutant CD59**—After transfection and geneticin selection, CD59 antigen was detected on the surface of cells stably transfected with wild type or with the mutant (Gln8/Gln18 or Asn5/Gln8/Gln18) constructs (data not shown). Comparison of results obtained with the mutant plasmids to that of wild type pcDNA3-CD59 revealed no consistent differences in the level of expressed anti-CD59 activity when compared with wild type CD59. Furthermore, we demonstrated that transposition of a variant glycosylation site (identified in HVS CD59) to human CD59 results in glycosylation at Asn5 without any apparent change in MAC inhibitory function when compared with wild type (Asn5-glycosylated) CD59.

**DISCUSSION**

In the present study, we show that the prominent N-linked glycosylation found in CD59 is not required for normal cell surface expression of the protein, nor does it contribute to its MAC-inhibitory function. Furthermore, we demonstrate that transposition of a variant glycosylation site (identified in HVS CD59) to human CD59 results in glycosylation at Asn5 without any apparent change in MAC inhibitory function when compared with wild type (Asn5-glycosylated) CD59.

In contrast to this report, various studies have suggested that glycosylation may be an important component of the com-

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2 R. P. Rother, unpublished data.
The data shown are of a single experiment and are representative of complement regulatory activity of human CD59. For example, recombinant soluble CD59 isolated from bacteria (which are incapable of N-linked glycosylation) showed markedly diminished MAC inhibitory activity, suggesting that one or more post-translational modifications may be required for normal function (21, 22). Based on data in the present study demonstrating that glycosylation is not required for MAC inhibition, it is likely that loss of complement regulatory function in bacterially produced CD59 is a result of improper folding or the deletion of the glycosylphosphatidylinositol anchor and not the absence of N-linked carbohydrate per se.

A partial loss of MAC inhibitory function was previously observed when purified erythrocyte CD59 was subjected to enzymatic deglycosylation, followed by membrane re-incorporation (10). Nevertheless, this solubilized and deglycosylated form of CD59 retained the capacity to specifically bind to human C8 and C9. These results suggested that the observed loss of function in the deglycosylated protein was related to a change in CD59 orientation or topology following re-incorporation into the membrane and not actual loss of a motif in the protein required for interaction with MAC. Consistent with this interpretation, recombinant CD59 expressed and deglycosylated in a heterologous cell line (Chinese hamster ovary cell) was observed to retain the human-selective MAC inhibitory function that is characteristic of human erythrocyte CD59, suggesting that the apparent species-selective function of CD59 is not determined by post-translational modification unique to expression in primate cells.

In contrast to studies suggesting that N-linked glycosylation contributes to the MAC inhibitory activity of CD59, one report suggested that cells transfected with recombinant CD59 containing a Ser → Ala mutation at residue 20 (to eliminate the Asn-linked glycosylation site) showed increased complement regulatory function when compared with cells transfected with wild type CD59 (23). Nevertheless, because the level of cell surface expression of each recombinant protein (mutant or wild type) was not reported, these data do not permit definitive conclusion as to the relative MAC inhibitory function of the Ser→Ala mutant. Furthermore, this study did not resolve whether this mutation resulted in glycosylation at CD59 residue Asn, a second potential site for N-glycosylation that is normally not glycosylated in wild type CD59.

It is still unclear why the motif for Asn-linked glycosylation in human CD59 is conserved in all homologous mammalian proteins for which sequence is now available, including primate (New World: owl monkey, squirrel monkey, and marmoset; and Old World: human, baboon, and African green monkey) (11–13) and rat (14). One possibility is that this glycosylation serves a function unrelated to MAC inhibitory activity. For example, CD59 has been shown to function as a ligand for the T cell accessory molecule CD2, and deglycosylated CD59 was found to exhibit diminished capacity to interact with lymphocyte CD2 (24–26). Alternatively, glycosylation at Asn may serve an ancillary function unrelated to any of the biological activities of CD59. For example, although we observed normal cell surface expression of the unglycosylated Gln/Gln mutant when expressed by transfection of cultured cells, the possibility remains that such glycosylation is normally required to stabilize the protein at the surface of blood cells during maturation and circulation in vivo.

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