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Membrane Cofactor Protein: Importance of *N*- and *O*-Glycosylation for Complement Regulatory Function[1]

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Membrane cofactor protein (MCP; CD46) is a type 1 membrane glycoprotein that inhibits complement activation on host cells. It also is a measles virus (MV) receptor, an adherence factor for group A *Streptococcus pyogenes*, and a cellular pilus receptor for pathogenic *Neisseria.* The amino terminus of MCP consists of four complement control protein (CCP) repeats, three of which (CCP-1, -2, and -4) possess *N*-glycans. Immediately following the CCP modules is an alternatively spliced region for extensive *O*-glycosylation (termed the STP domain). Previous studies established that the *N*-glycan of CCP-2 is essential for MV binding and infection and that the splicing variants of the STP domain not only affect MV binding and fusion, but also differentially protect against complement-mediated cytolysis. In this report, we dissect the role of these carbohydrates on complement regulatory function. We constructed, expressed, and characterized proteins deleting these carbohydrates. For MCP-mediated protection against cytolysis, the *N*-glycans of CCP-2 and -4 were necessary, the STP segment influenced but was not essential, and the *N*-glycan of CCP-1 was not required. In addition, the rate and magnitude of cell surface cleavage of C4b to C4c and C4d by MCP and factor I correlated with cytoprotection. These studies expand the structure-function understanding of the active sites of MCP and elucidate an important role for carbohydrates in its function, a finding consistent with their conservation in the MCP of other species. *The Journal of Immunology,* 1998, 161: 3711–3718.

Membrane cofactor protein (MCP[3]; CD46) is a regulator of complement activation expressed by most human cells and tissues (reviewed in Ref. 1). MCP protects the cell on which it resides, serving as a cofactor for the plasma serine protease factor I to inactivate deposited C4b and C3b. In addition, MCP is a receptor for several pathogens including measles virus (MV) (2–4), group A *Streptococcus pyogenes* (5), and pathogenic *Neisseria* (6). MCP also is involved in reproduction. For example, it is expressed on placental trophoblast and the inner acrosomal membrane of human spermatozoa (7–9), possibly having a role in both fertilization (10) and habitual abortion (11). Finally, MCP (12) and related human complement regulators are undergoing evaluation for therapeutic use as soluble inhibitors of complement activation and are being engineered into transgenic animals to prevent the hyperacute graft rejection that accompanies xenotransplantation (reviewed in Refs. 13 and 14).

Most cells express MCP as a family of four isoforms generated by alternative splicing at two sites (Refs. 15 and 16; reviewed in Ref. 1). The amino terminus is identical for all isoforms, consisting of four of the ligand-interacting modules termed complement control protein (CCP) repeats. Functional sites for C3b and C4b interactions have been mapped to CCP-2, -3, and -4 (17, 18). *N*-linked glycosylation occurs in CCP-1, -2, and -4 (19). Following the repeats is the alternatively spliced *O*-glycosylated region that is enriched in serines, threonines, and prolines (STP domain). A juxtamembranous segment of 12 amino acids of unknown function terminates the extracellular portion of MCP. Hydrophobic transmembrane and alternatively spliced cytoplasmic domains (Cyt-1 or Cyt-2) complete the carboxyl terminus. The four isoforms are termed BC1, BC2, C1, and C2 to denote their STP (i.e., BC or C) and cytoplasmic tail (one or two) content. Rarer isoforms have been described (16).

Although the binding site(s) for group A *Streptococcus* and *Neisseria* have not been mapped, CCP-1 and -2 are critical for MV binding and infection (18, 20–23). Additionally, the *N*-glycan of CCP-2 is essential for MV binding and infection, whereas those of CCP-1 and -4 are of minor importance (21, 24–26). It has been suggested that, rather than contributing to specific binding, the *N*-glycan of CCP-2 maintains the conformational angle or reduces flexibility between CCP-1 and -2, thereby providing a more rigid docking site for MV hemagglutinin (21). Because of the above findings and evidence that isoforms with a smaller STP domain have a diminished ability to protect against complement attack (18, 27), we sought to examine the impact of these carbohydrate modifications on complement regulatory function by creating, expressing, and evaluating mutants deleted of these sites.

In the present study, we prepared stably transfected glycan deletion mutants in Chinese hamster ovary (CHO) cells. Initially, we utilized cell lysates of these mutants to assess C3b and C4b cofactor activity and ligand binding. Next, employing clones bearing equivalent expression levels of control or mutant MCP, we evaluated cytoprotection and C4b fragment cleavage on the cell surface. We determined that the *N*-glycans of CCP-2 and -4 and the STP domain were involved in MCP-mediated cytoprotection, a finding consistent with the evolutionary conservation of these carbohydrate sites.

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[3] Abbreviations used in this paper: MCP, membrane cofactor protein; MV, measles virus; CCP complement control protein; STP, serine/threonine/proline-enriched segment; CHO, Chinese hamster ovary; TMB, 3,3′,5,5′-tetramethylbenzidine; NHS, normal human serum.

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Materials and Methods

Construction of cDNA mutants, expression, and isolation of clones

Four mutants of the BC1 isoform were constructed. For three constructs a glutamine (Q) replaced the asparagine (N) in CCP-1, -2, and -4 and were termed NQ1, NQ2, and NQ4 (25). The fourth construct, termed ΔSTP, deleted the STP domain by ligating two PCR-derived segments of BC1 in the expression plasmid pHβApr1.neo (27). These two segments coded for the signal peptide through the end of CCP-4 (nucleotide residues 42–1000) and the region coding for the "undefined" segment through the end of the cytoplasmic tail (nucleotides 1088–1518) (see GenBank MCP-BC1 cDNA, accession no. X59405). All cDNA clones were sequenced in their entirety to verify fidelity.

Transfection was performed per manufacturer's directions using Lipofectin (Life Technologies, Gaithersburg, MD) in CHO K1 cells. Transfected cells were selected and maintained in Ham's F-12 medium supplemented with 10% FCS and 0.5 mg/ml geneticin. CHO mutant clones were obtained by limiting dilution and were selected for expression levels comparable to wild-type clone BC1 (designated as 23-9, as described in Ref. 27).

Flow cytometry

Flow cytometric analysis was performed as described (27). MCP mAbs utilized were as follows: TRA-2-10 (28), which binds to an epitope in CCP-1 (20); M75, which binds to an epitope in CCP-2 (provided by Tsukasa Seya, Osaka, Japan) (18); and GB24 (29), which binds to a complement functional epitope in CCP-3/4 (17). A rabbit polyclonal Ab to MCP was kindly provided by CytoMed (Cambridge, MA) (27).

Cell lysates and quantification

Cells were lysed (2×10^7 cells/ml) in 1% Nonidet P-40, 0.05% SDS in TBS (10 mM Tris, pH 7.2, 150 mM sodium chloride) with 2 mM PMSF for 15 min at 4°C, followed by centrifugation in a microcentrifuge at 12,000 × *g* for 10 min. Supernatants were collected and MCP quantified by ELISA as previously described (17), except as noted below. Briefly, MCP mAb TRA-2-10 was coated at 5 μg/ml in TBS overnight at 4°C in microtiter wells (Nunc modules, Fisher Scientific, St. Louis, MO). Wells were blocked for 1 h at 37°C with 1% BSA and 0.1% Tween-20 in TBS and rinsed in wash buffer (TBS with 0.05% Tween-20). Cell lysates were prepared over nearly a log of dilutions in dilution buffer (wash buffer with 4% BSA and 0.25% Nonidet P-40), applied to wells along with an MCP standard, incubated for 1 h at 37°C, and rinsed for 2 min three times with wash buffer. Next, rabbit anti-MCP antiserum was diluted 1:7000 in dilution buffer, applied for 1 h at 37°C, and similarly washed. Horseradish peroxidase-coupled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was incubated for 1 h at 37°C followed by similar washing. Detection was made using the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Pierce, Rockford, IL). Optical density (630 nm) was measured in an ELISA reader.

Western blot analysis

For Western blot analysis, cell lysates of CHO transfectants (200,000 cell equivalents/lane) were analyzed on a 10% SDS-PAGE and transferred to nitrocellulose as previously described (27).

Confocal microscopy

Cells were treated identically as described for flow cytometry (27) with the exceptions noted below. Briefly, cells were grown to ~70% confluency, collected by brief trypsinization, washed in PBS, resuspended at 5×10^6 cells/ml in 1% FCS in TBS at 4°C, and 100 μl was placed in wells of a V-bottom microtiter plate. MCP mAb TRA-2-10 IgG was added at 5 μg/ml for 30 min at 4°C. Cells washed in 1% FCS, and FITC-conjugated $F(ab')_2$ donkey anti-mouse IgG (Jackson ImmunoResearch) was added for 30 min at 4°C. Following washing, cells were fixed in 0.5% paraformaldehyde, collected by cytospin onto microscope slides, and mounted. Fluorescence images were collected with a ×63 oil objective (NA 1.4) using a Bio-Rad (Hercules, CA) MRC 1024 confocal laser-scanning adaptor attached to a Zeiss Axoplan upright microscope. The 488-nm lines of an argon-krypton laser were used for excitation, and the emitted light was filtered through a 522-nm long-pass filter cube and detected by respective photomultipliers. Images, each of which is 512 × 512 pixels, were recorded with Bio-Rad Lasersharp software. An image was averaged by two successive frames, and a stack of 8 to 12 such images was obtained for each cell.

Cofactor assay

This assay utilizes biotinylated ligands (C3b and C4b). The fragments of the cleavage reaction, resulting from addition of factor I with MCP, were assessed by Western blot analysis following electrophoresis (10% reducing SDS-PAGE). C3b and C4b (Advanced Research Technologies, San Diego, CA) were biotinylated utilizing a 50× molar excess of EZ-Link Sulfo NHS-LC-biotin (Pierce) per the manufacturer's directions. The sample was dialyzed with a Microcon 30 unit (Amicon, Beverly, MA), and aliquots were stored at −70°C. To optimize the signal, serial dilutions of the reduced biotinylated ligand (25 mM DTT) were evaluated on a 10% SDS-PAGE gel and then transferred to nitrocellulose for Western blot analysis (27). Membranes were rinsed in TTBS (TBS with 0.05% Tween-20) for 2 min and probed with 1:1500 dilution of ExtrAvidin-horseradish peroxidase (Sigma, St. Louis, MO) for 1 h at 37°C with rotation. Blots were washed three times with TTBS for 5 min each with rotation. Using a clean dish, chemiluminescent substrate (SuperSignal; Pierce) was incubated for 1 min with the blot. The blot was wrapped in plastic wrap and x-ray film exposed for varying amounts of time followed by film development. The dilution that produced the optimal signal to noise ratio was utilized.

For the cofactor assay, all dilutions were prepared in low salt buffer (10 mM Tris, pH 7.2, with 25 mM sodium chloride, 1% Nonidet P-40, and freshly added 2 mM PMSF). C3b or C4b were diluted as determined above. Factor I (Advanced Research Technologies) was added (100 ng), and cell lysates (varying from 2.5 to 5×10^8 MCP/assay) were added in a total of 15 μl. Following an incubation at 37°C for 1.5 h, an equal volume of 2× reducing buffer was added, the samples heated to 95°C for 3 min, and SDS-PAGE/Western blotting performed as described above. X-ray films were scanned by using a laser densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ) and analyzed with GELscan software (Pharmacia). The generation of the C4d fragment was monitored and adjusted relative to the input as measured by the γ-chair fragment of C4b. Mutants were compared with wild-type MCP.

Ligand binding ELISA

C3b and C4b (Advanced Research Technologies) were coated at 5 μg/ml in TBS overnight at 4°C in microtiter wells (Nunc modules; Fisher Scientific) as described (27).

Briefly, coated wells were blocked 1 h at 37°C with 1% BSA and 0.1% Tween 20 in TBS and washed in low salt ELISA buffer (LSEB) consisting of 10 mM Tris (pH 7.2), 25 mM sodium chloride, and 0.05% Tween 20. Lysates, quantified for MCP by ELISA (see above), were diluted to equivalent quantities over nearly a log in LSEB, and incubated 2 h at 37°C. Following washing in LSEB, rabbit antiserum to MCP was diluted 1:2500 in LSEB and 4% BSA/0.25% Nonidet-P40, and incubated for 1 h 37°C. After washing, horseradish peroxidase-coupled donkey anti-rabbit IgG was added and incubated 1 h 37°C, and the wells were washed. Detection was made utilizing TMB, and optical density (630 nm) was measured in an ELISA reader.

Cytoprotection assays

Clones obtained by limiting dilution (see above) possessing equivalent levels of MCP mutants were isolated and employed in cytoprotection assays as described previously (27). Briefly, 10,000 cells/well were plated overnight in 96-well microtiter plates. The medium was removed and rabbit anti-hamster Ab (IgG fraction; Sigma) was added (13 mg/ml) for 30 min at 4°C. Ab was removed and serum (diluted in gelatin veronal buffer) was added for 1 h at 37°C. Wells were washed twice in Dulbecco's PBS, and normal medium was added. Cells were grown for 48 h and assessed with the CellTiter 96 kit per manufacturer's directions (Promega, Madison, WI). Assays were performed in quadruplicate on at least three separate occasions. Untreated, Ab-only, and serum-only controls were performed with each assay. Normal human serum (NHS) and C6-depleted serum (utilized in C4 deposition studies) were obtained from Quidel (San Diego, CA).

C4b fragment deposition

These studies were performed as previously described (27). Briefly, cells were challenged identically as for the cytoprotection assays except that C6-depleted serum was utilized to prevent cytolysis. In addition, for kinetic analysis, the C6-depleted human serum was incubated for the time points indicated. mAbs to C4c and C4d (20 μg/ml) were incubated with these sensitized cells for 30 min at 4°C. Cells were washed and incubated with FITC-conjugated goat anti-mouse IgG for 30 min at 4°C followed by washing. Cells were fixed in 0.5% paraformaldehyde, and FACS analysis was performed.

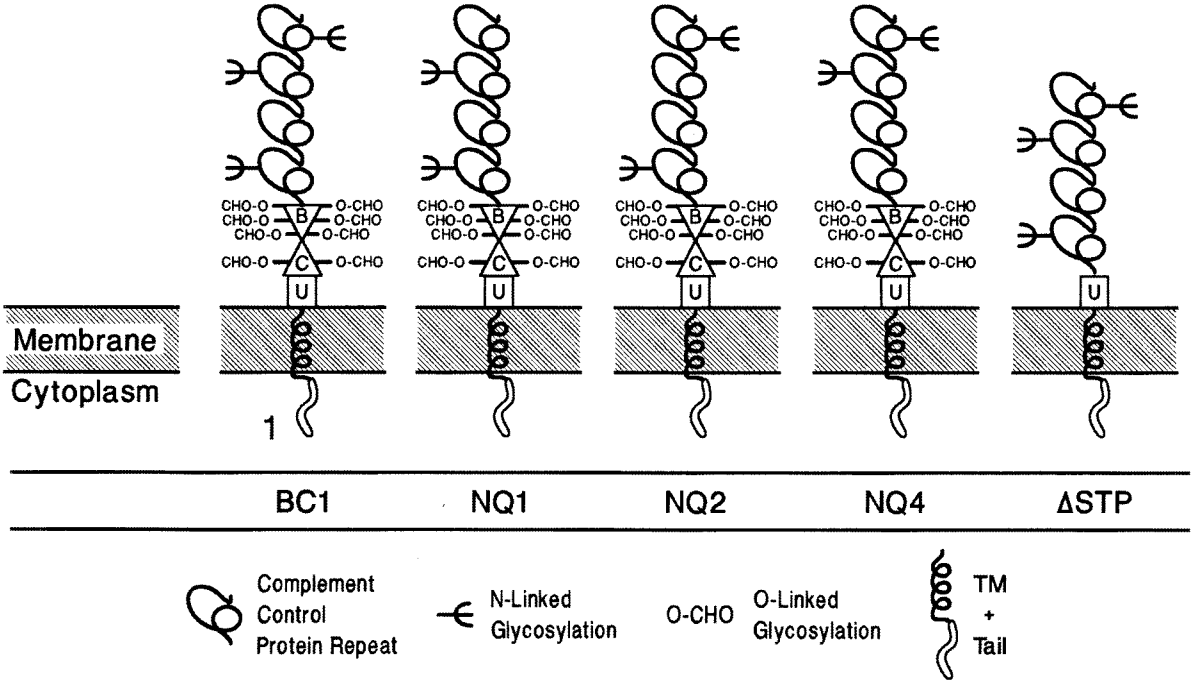

FIGURE 1. Schematic diagram of native MCP and the four glycan deletion constructs evaluated in this report. Common to all are: 1) four of the repeating, ligand binding motifs called CCP repeats; 2) the small juxtamembranous segment of unknown functional significance ("U"); 3) the membrane-spanning, hydrophobic domain; and 4) the intracytoplasmic tail (number 1). The NQ constructs substituted a glutamine (Q) residue for the asparagine (N) in CCP-1 (termed NQ1), in CCP-2 (NQ2), and in CCP-4 (NQ4). For the ΔSTP construct, this region was deleted entirely.

Results

Characterization of mutant pooled and cloned transfectants

To analyze the importance of *N*- and *O*-linked glycosylation on MCP complement regulatory function, mutants were constructed by using site-directed mutagenesis to delete each of these sites (Fig. 1). The motif for *N*-linked glycosylation (N-X-S/T, with X being any amino acid except proline (30)), was mutated such that a glutamine (Q) was substituted for the asparagine (N) in CCP-1, -2, and -4 and termed NQ1, NQ2, and NQ4, respectively. To assess the role of *O*-linked sugars, a mutant deleting the STP domain was created (termed ΔSTP). Following the stable transfection of mutant and control cDNAs into CHO cells, expressed MCP and mutant protein were assessed with polyclonal and/or monoclonal anti-MCP Abs by FACS and Western blotting. Subsequently, clones with equivalent levels of expression were isolated and evaluated.

As shown in the Western blot of Figure 2, the BC1 isoform and glycan deficient mutants displayed the expected relative mobilities. NQ2 and NQ4 possessed relatively faster mobilities consistent with the loss of one *N*-glycan group, whereas the ΔSTP mutant demonstrated the fastest mobility (as expected, since it lacks all *O*-glycosylation). The somewhat faster mobility of NQ1 vs NQ2 and NQ4 was noted previously and likely results from a conformational effect since the mobility of these forms is equivalent following reduction (25).

Clones were derived by limiting dilution and selected for stability and equivalency of MCP expression by FACS using a polyclonal anti-MCP antiserum (lower part of Fig. 2). The clones utilized were designated as 23-9 (BC1), NQ1-8, NQ2-5, NQ4-12 and ΔSTP-30. Next, further comparative evaluations for protein stability and expression patterns of each of these clones were undertaken. First, epitope sites were evaluated by FACS with several monoclonal anti-MCP Abs (data not shown). The clones selected exhibited similar MCP expression levels and retained epitopes represented by the mAb: 1) TRA-2-10, which binds a site in CCP-1 and inhibits MV binding (20); 2) M75, which binds to a complement regulatory and MV functional site in CCP-2 (18, 20), and 3) GB24, which binds to a complement regulatory site in CCP-3/-4 and blocks C3b/C4b binding (17). The FACS profiles for the three mAb were similar, suggesting that these mAb bind to a peptide epitope unaltered by the lack of glycans (data not shown).

Second, clones were exposed to human serum (1:8 dilution) for 1 h at 37°C and then lysed. Western blot analysis with an anti-MCP polyclonal Ab demonstrated no significant differences in pattern of electrophoretic migration or protein quantity of treated and untreated cells (data not shown).

Third, to examine cell surface distribution, confocal microscopy was performed on clones utilizing an anti-MCP mAb (TRA-2-10) (Fig. 3). This examination revealed a similar and uniform MCP surface distribution for all clones. In addition, clones "challenged"

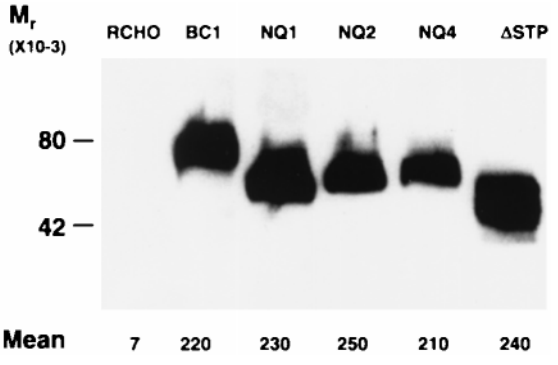

FIGURE 2. Western blot analysis of control and mutant transfectant lysates. FACS means of the isolated clones are listed in the lower portion of the figure. Nomenclature as in Figure 1. Rabbit polyclonal antiserum to MCP was utilized as a probe. RCHO, a Chinese hamster ovary (CHO) cell line transfected with MCP construct in the reverse orientation.

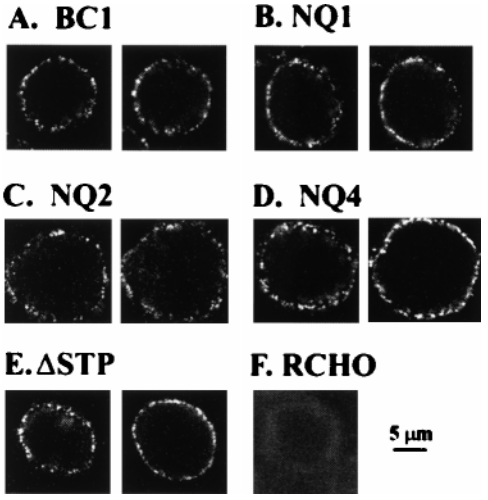

FIGURE 3. Confocal microscopy of MCP or control CHO cell clones utilizing a mAb to MCP (TRA-2-10) and $(Fab')_2$ FITC-anti mouse IgG. (Scale bar = 5 μm.) Two midline frames of each clone are presented.

by Ab and C6-deficient human serum (see below) maintained this pattern (data not shown).

NQ4 and ΔSTP cell lysates exhibit decreased cofactor activity

Initial comparisons of C3b and C4b cofactor activity were performed utilizing cell lysates of transfectant pools. As indicated in Figure 4, NQ4 and ΔSTP lysates had reduced C3b and C4b cofactor activity, whereas NQ1 and NQ2 were equivalent to BC1 (wild type) in C3b activity, but demonstrated a modest decrease in C4b cofactor activity. These studies suggested that the *N*- and *O*-glycosylation domains may be important for regulatory function. To further examine this possibility and to determine whether alterations in ligand binding accounted for these functional differences, we performed C3b and C4b binding studies.

Glycosylation mutants bind C3b and C4b similarly to wild type

For ligand binding, an ELISA was utilized in which C3b or C4b was adsorbed to wells and dilutions of quantified transfectant lysates were evaluated (Fig. 5). All mutants retained ligand binding capability similar to wild-type BC1. As noted previously (27), there was a modest, but reproducible, preferential binding by wild-type BC1 to C4b vs C3b that was maintained by all mutants except

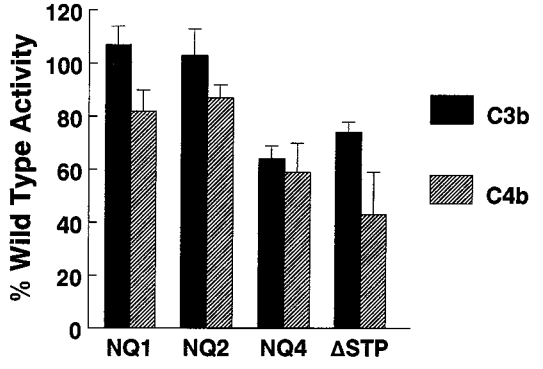

FIGURE 4. C3b and C4b cofactor analysis of mutants as compared with wild-type MCP. Transfectant cell lysates were quantified by ELISA, incubated with factor I and biotinylated C3b or C4b for 90 min at 37°C, and separated on reducing SDS-PAGE; a chemiluminescent Western blot analysis was performed utilizing horseradish-avidin conjugate. Laser densitometry was utilized to compare wild-type to mutant cleavage patterns.

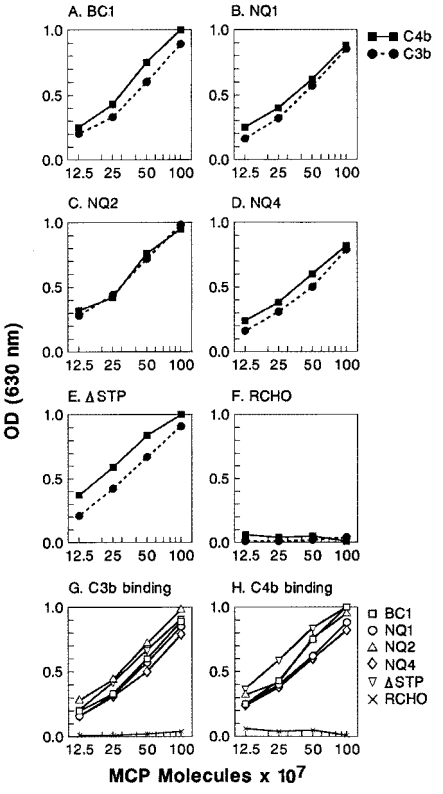

FIGURE 5. Comparison of C3b and C4b binding by control and mutant transfectants. Cell lysates were prepared for stably transfected CHO cells and quantified in an ELISA. Increasing amounts of cell lysates were incubated on C3b- and C4b-coated wells. *A–F*, Binding of each sample to C3b and C4b; *G* and *H*, comparison of the binding by all to C4b or C3b. A representative experiment of three is shown.

NQ2 (see Fig. 5, *A–E*). NQ2 also showed a slight elevation in binding to both ligands, but more so to C3b. There was a consistent pattern of slightly enhanced binding by NQ2 and slightly less binding by NQ4 to both C3b and C4b. We concluded that, in this solid phase assay using solubilized cell lysates, deletion of the *N*- or *O*-linked glycosylation sites had minimal influence on ligand binding. Retention of ligand binding despite partial loss of cofactor activity as observed for NQ4 and ΔSTP is consistent with previous findings that binding and cofactor activity are separable (17, 31).

N-glycosylation domains in CCP-2 and -4 are critical for cytoprotection

The clones profiled in Figure 2 were utilized in cytoprotection assays. The cells were treated with a polyclonal Ab to hamster cell surface Ags followed by incubation with NHS as the source of complement. As expected and established previously, the classical pathway mediates cytoinjury to CHO cells in this system (27, 32, 33).

Figure 6 compares cytoprotection by control and mutant clones over a range of serum dilutions. The NQ1 mutant was similar to wild type, suggesting little effect by the *N*-glycan in CCP-1, consistent with previous results in which CCP-1 was deleted without altering ligand binding and cofactor activity (17, 18). However, mutants NQ2 and NQ4 demonstrated a marked decrease in their cytoprotective capability with an activity profile very similar to the RCHO control cells (CHO cells transfected with MCP construct in the reverse orientation). The mutant deleted of the STP region exhibited an intermediate capacity for cytoprotection.

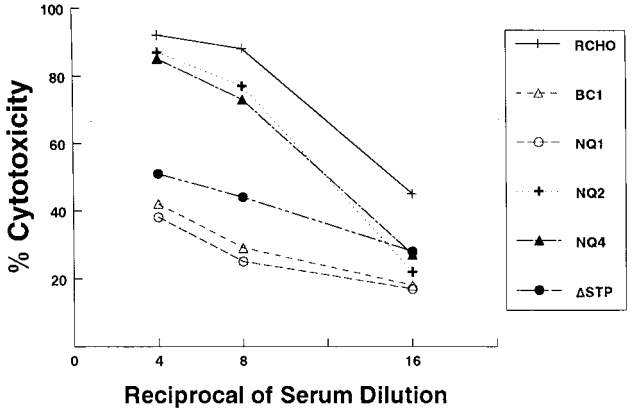

FIGURE 6. Comparison of cytoprotection by control and mutant transfectant clones bearing equivalent expression levels of MCP. Cell clones or control CHO were assessed in a classical C pathway-mediated system employing rabbit Ab, dilutions of human C (NHS), and Promega CellTiter 96 kit. Percent cytotoxicity was calculated based upon values of untreated controls (Ab alone and NHS alone controls were equivalent to untreated). The SE of the mean among quadruplicates was <7%. Shown is a representative experiment of three.

N- *and* O- *glycosylation deletion mutants have diminished C4 cleavage on the cell surface*

The differences in the cytoprotective ability of the glycosylation deletion mutants implied differential potencies of the mutant proteins to serve as cofactors for the factor I-mediated cleavage of C3b and C4b. Also, since differences had been noted in cofactor assays employing cell lysates (see Fig. 4), we assessed cofactor function on the cell surface. For this investigation, we employed a nonlytic system in which clones were treated with the same anti-CHO cell Ab, but C6-deficient human serum was utilized. The cleavage of C4b was monitored by FACS with mAb against the cleavage fragments of C4b. Cleavage of C4b by MCP releases a large fragment (C4c), whereas the smaller fragment (C4d) remains covalently attached to the cell membrane. As C4b is cleaved, the quantity of C4c is reduced while the C4d level remains constant. Thus, a comparison of cell surface C4c vs C4d by FACS permits a quantitative assessment of the kinetics of C4b cleavage.

Representative histograms comparing C4c vs C4d on the cell surface are presented in Figure 7. On control cells that do not express MCP (Fig. 7*B*), the quantities of C4c and C4d were coincident, indicating that cell-bound C4b was not cleaved and establishing that C4b binding protein or other regulatory proteins had no activity in this regard. All cells expressing MCP possessed C4b cofactor activity, since there was a reduction of cell-bound C4c relative to C4d (Fig. 7, *C–G*). A comparison of the relative mean fluorescence intensity for these two C4 fragments is presented in Table I. The relative intensity values were the lowest for wild-type (BC1) and NQ1 (indicating the highest C4b-cleaving activity) followed by NQ4, NQ2, and ΔSTP. This pattern parallels results obtained by the cytotoxicity experiments, except that ΔSTP had less cofactor activity than anticipated.

Table I. *C4 fragment deposition on complement-treated control and mutant cells*[a]

Cells	FACS Mean		Ratio, C4c/C4d
	Anti-C4c	Anti-C4d	
RCHO+	1180	1065	1.10
BC1	200	1111	0.18
NQ1	198	1265	0.16
NQ2	439	1207	0.36
NQ4	276	1146	0.24
ΔSTP	544	1328	0.40

[a] Conditions are as described in the legend to Figure 7. FACS means are from duplicates. A representative experiment of three is presented.

Kinetic analysis of C4b cleavage

To further assess these differences in cofactor activity, we examined the cleavage patterns after 10, 20, and 60 min of complement exposure (Fig. 8). These data indicate that MCP-dependent cofactor activity begins concomitant with C4b deposition. Rapid, incremental cleavage then occurred for at least 20 min for wild type BC1 as well as the mutants. By 20 min, ~50% of the C4b had been cleaved in the case of BC1 and NQ1, whereas less cleavage took place for NQ4, NQ2, and ΔSTP. At the final time point of 60 min, 25 to 35% of the C4b had not been cleaved to C4c and C4d in wild type and all mutants except ΔSTP, in which case 50% remained as C4b.

Discussion

Several previous studies have suggested that MCP carbohydrates are functionally relevant (21, 24, 25, 27, 34, 35). The *N*-glycans in CCP-2 are important for MV binding and infection (21, 24, 25), whereas the STP domain may have effects on fusion as well as

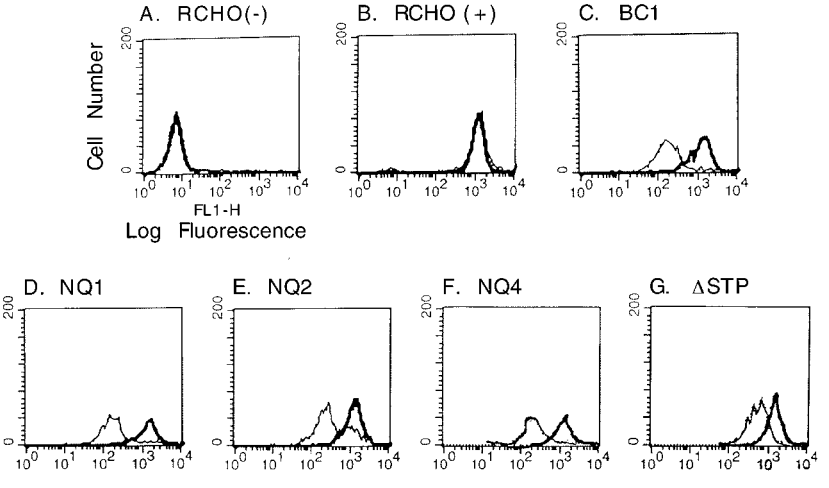

FIGURE 7. Flow cytometric analysis of the C4b cleavage fragments of control and mutant clones. The light line shows C4c, and the dark line shows C4d. *A* and *B*, RCHO control (transfected with MCP in reverse orientation) was evaluated without (−) and with (+) Ab to CHO. For *B*, the "unprotected" CHO control cells demonstrate overlapping C4c and C4d patterns, indicating no C4b degradation. In this assay, cells were sensitized by incubation with Ab followed by addition of a 1:8 dilution of C6-depleted human serum. Three separate experiments were performed with each condition in duplicate. Representative histograms are shown. See Table I for mean fluorescence intensities.

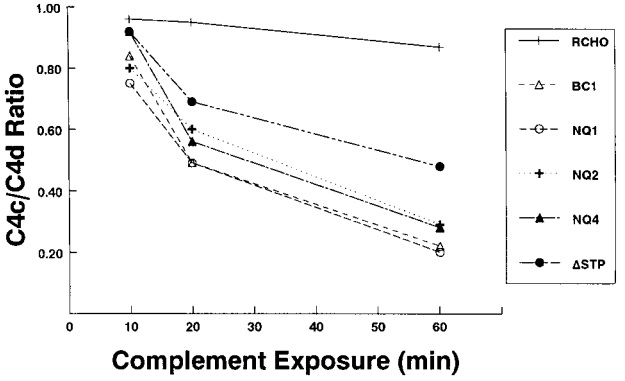

FIGURE 8. Kinetic evaluation of C4b cleavage by control and mutant clones. Experimental conditions similar to that in Figure 7. FACS means are an average of duplicates. A representative experiment of three is presented.

binding (34, 35). In addition, the alternatively spliced *O*-glycosylation domain influences complement regulatory function in that higher m.w. (BC) isoforms protect better against the classical pathway than the less *O*-glycosylated, lower m.w. C isoforms (27). Finally, tissue-specific expression of MCP isoforms suggests functional relevance of the STP domain, e.g., BC isoforms predominate in the kidney and salivary gland, whereas C isoforms predominate in the brain (34, 36, 37).

The goal of the present study was to further examine these glycan modifications in MCP, and in particular assess the importance of carbohydrates as they relate to complement regulatory function. The initial C3b and C4b cofactor assays suggested that glycosylation played a role in regulation since both NQ4 and the ΔSTP mutants demonstrated reduced activity. However, ligand binding studies indicated that all mutants bound both C3b and C4b similarly to wild type. To examine these potentially discrepant observations, we assessed MCP function in a more biologically relevant assay system.

A significant finding of these subsequent experiments was that the *N*-glycans of CCP-2 and -4 were critical for the cytoprotective activity of MCP. The deletion of either nearly abrogated this inhibitory capability and, thus, was a much more striking functional deficiency than would have been predicted from the ligand binding and cofactor assays employing solubilized cell extracts. This activity profile was explained, in part, by a decreased ability for C4b cleavage in the fluid phase (Fig. 4) as well as on the cell surface (Fig. 7, Table I). These results are consistent with previous data indicating that CCP-2 and -4 are required for complement regulatory function (17, 18). The deletion of the *N*-glycan in CCP-1 did not diminish the cytoprotective function of MCP, a result also consistent with previous findings that 1) CCP-1 does not contribute to complement inhibitory function (17, 18) and 2) it is spliced out in new world primates (22). Finally, it is worth noting that, in these cytoprotection experiments, cleavage was strictly MCP dependent with no detectable activity by C4b-binding protein since non-MCP expressing CHO cells did not demonstrate cleavage of C4b (see Fig. 7).

Loss of efficient cytoprotective capacity by glycosylation mutants could be explained by other reasons such as failure of the mutant protein to be transported to the cell surface or to gross conformational alterations. Multiple lines of evidence suggested that these did not occur. First, we were able to obtain stable clones of mutants and to select for equivalent expression levels utilizing FACS. Second, Western blots bearing similar cell equivalents revealed appropriate m.w. sizes and similar quantities. Third, there were no alterations in the interactions of mutants with peptide-specific mAbs to MCP. Fourth, Western blot profiles of cells exposed to human serum were identical to untreated controls, demonstrating no serum-dependent degradation of MCP. Fifth, confocal microscopy revealed similarly uniform distribution patterns of MCP protein for all clones (also true of cells "challenged" by Ab and complement (C6-deficient serum)). Last, ligand binding assays employing solubilized transfectant lysates demonstrated similar activity of all mutants as compared with wild-type MCP.

The necessity of *N*-glycans for function is variable among complement regulatory proteins. For example, the deletion of *N*-glycans from C4b-binding protein, decay accelerating factor (DAF, CD55), and CD59 did not diminish complement inhibitory activity (38–40). Consequently, it has been suggested that the *N*-glycans of these proteins may serve in other capacities. Specifically, the single *N*-site of CD59 is conserved in all homologous mammalian proteins examined to date and may be a site of interaction with the T cell accessory molecule CD2 (40). On the other hand, *N*-glycans are critical for the function of many other proteins including the *N*-linked carbohydrates of Igs that serve a variety of effector functions including complement activation and ligand interaction) (reviewed in Refs. 41 and 42). In addition, for the IFN-γ receptor, inhibition of *N*-linked glycosylation or modulation of carbohydrate processing did not prevent receptor transport to the cell membrane, yet blocked ligand binding thereby completely abrogating receptor function (43).

An indication of the importance of *N*-glycans in MCP is suggested by their evolutionary conservation across species (Fig. 9). Recently, MCP sequence data have become available for African green monkey (Vero) (44), guinea pig (45), pig (46, 47), and several old and new world monkeys (22). The *N*-sites in CCP-1, -2, and -4 are conserved in human, Vero, and all five characterized species of old world monkeys. Interestingly, MCPs of new world monkeys retain the *N*-linked sites in CCP-2 and -4, yet delete CCP-1 entirely. Deletion of this domain by new world monkeys may have afforded protection against a potentially lethal microorganism such as MV. Three additional *N*-sites were possible in Vero, although enzymatic treatment suggested these may not be utilized (44), which also may be the case for the other old world monkeys. In 13 of the 14 species examined, the location of the *N*-site of CCP-4 was uniformly conserved. The one exception was guinea pig MCP, which also possessed an *N*-glycan in CCP-4, but at a slightly different locale. Such evolutionary conservation of these posttranslational modifications, in most cases of the identical sites, strongly suggests they play important biologic roles.

Deletion of the STP segment produced an intermediate loss of cytoprotective activity. This finding was not predicted by results of the cell-bound and fluid phase cofactor assays, which indicated that ΔSTP was the least effective cofactor among the mutants. One explanation may be that these tests assess different parameters in that cytotoxicity studies measured cell survival, whereas cell lysates and cell surface C4c content exclusively gauged cofactor activity. Also of note, the level of cytotoxicity and the C4c/C4d ratio for ΔSTP was comparable to that observed previously for the MCP-C1 isoform (which has a smaller and less glycosylated STP domain) (27). The interpretation of this earlier study was that the larger BC segment augmented the ability of MCP to act as a cofactor for C4b degradation on the cell surface (27) and is consistent with the present results of entirely eliminating the STP domain. It should be recalled that more than 95% of the population express predominantly the higher (BC) glycosylation forms of MCP, whereas only 5% predominantly express the C isoforms (48). Taken together, these studies strongly indicate that the BC isoforms protect better against the classical pathway than C isoforms.

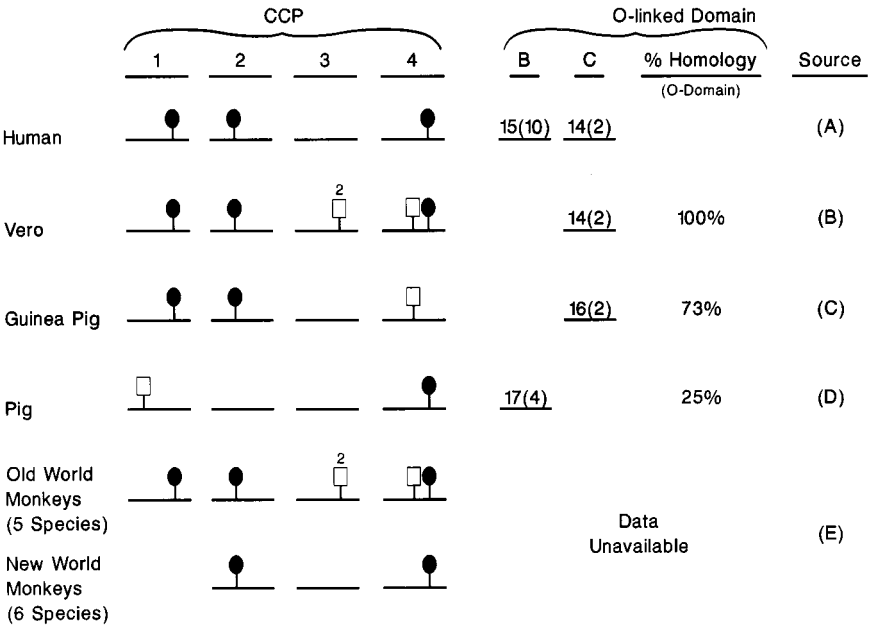

FIGURE 9. Comparison of sites of *N*- and *O*-glycans of MCP for all species with published sequences. The numbers beneath STP-B and -C segments of the *O*-linked domain represent the total number of amino acids per segment and inside parentheses are the number of serine/threonine residues. The dark circles represent the *N*-linked glycosylation sites in the human sequence. The open squares are *N*-linked sites not found in human MCP. References used are as follows: 19 for A; 44 for B; 45 for C, 46 and 47 for D; and 22 for E. For the primate data (22), authors indicated conservation of the *O*-linked domain but did not present the sequences.

Previous studies also indicated that the STP domain of DAF was functionally important. Deletion of this segment produced a complete loss of decay accelerating activity that was reestablished by re-inserting a similarly sized, but not *O*-glycosylated, segment from a noncomplement protein (39). These results point out a role for the STP region serving as a nonspecific spacer, perhaps projecting the CCP functional domains away from the plasma membrane. In our investigation, there was a moderate decrease in, but not a complete loss of, function. It is possible that the juxtamembranous segment (the "undefined" domain of 12 amino acids) may account for these differences.

The *O*-glycosylated region in MCP of the three other species available revealed that all possessed at least one domain for *O*-glycosylation following the CCP region. Vero MCP had a 14-residue segment identical to the STP-C domain of human MCP, guinea pig a 16-amino acid segment with 73% identity to the human C domain, and pig MCP a 17-amino acid segment with 25% homology to the B domain. Additional cDNA clones and RT-PCR in these species will be required to establish if this segment is alternatively spliced in a similar fashion to humans.

Our study and those of many others indicate that, in a classical pathway mediated system, large amounts of C4b can be deposited in a few minutes (49). The kinetic analysis of the cofactor activity of MCP mutants indicated that: 1) C4b cleavage began on the cell surface concomitant with deposition; 2) the cleavage pattern was linear for 20 to 30 min, with most of this activity occurring within this time frame; 3) substantial quantities (~25%) of C4b remained as such at 60 min, even with wild-type MCP; 4) surprisingly, there was no cleavage of C4b on the non-MCP expressing CHO control cells by serum C4b-binding protein; 5) in this system, the rate of C4b deposition far exceeds the rate of its cleavage, suggesting limits on the cytoprotective capabilities of regulatory proteins in Ab-mediated syndromes of autoimmunity; and 6) the ratio of deposited C4b ($6-8 \times 10^5$) to MCP (0.5×10^5) is estimated at greater than 10 to 1; thus, to cleave 75% of the C4b to C4d and C4c requires an interaction of each MCP with multiple C4bs.

The majority of membrane proteins of eukaryotic organisms are glycosylated. These posttranslational modifications play key roles in biologic functions (30, 50). For MCP, the discovery that the *N*-glycans of CCP-2 and -4 do not directly participate in ligand binding and only modestly reduce cofactor activity, but substantially influence cytoprotection, provides an instructive example of how these carbohydrate moieties may promote enhanced biologic activity at the cell surface that could not have been appreciated by studying either the isolated protein or solubilized MCP cell extracts. Indeed, the differences in activity point to additional biologic interactions not evaluated in the above experiments. For example, carbohydrates may facilitate the movement or clustering of MCP on a cell membrane that could assist its interactions with ligand, possibly in association with a convertase. Our findings of the necessity of *N*-glycans for function coupled with their evolutionary conservation across species establishes important biologic roles for these modifications in MCP.

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