Phospholipid-anchored and Transmembrane Versions of Either Decay-accelerating Factor or Membrane Cofactor Protein Show Equal Efficiency in Protection from Complement-mediated Cell Damage

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Summary

Decay-accelerating factor (DAF) is a glycosyl-phosphatidylinositol (GPI)-anchored membrane protein that protects cells from complement-mediated damage by regulation of the C3 convertase. To investigate the role of the GPI anchor in the function of DAF, the cDNA encoding human DAF was expressed by transfection in Chinese hamster ovary (CHO) cells. Testing of these DAF transfectants in an antibody plus human complement–mediated cytotoxicity assay demonstrated that DAF protects these cells from cytotoxicity, and that the level of protection increases with expression of surface DAF. A cDNA construct encoding a transmembrane version of DAF (DAF-TM) protects CHO transfectants from cytotoxicity with equal efficiency to DAF. This DAF-TM construct used the TM and cytoplasmic domains of membrane cofactor protein (MCP); an alternate TM version of DAF constructed with the TM and cytoplasmic domains of HLA-B44 showed equivalent protection. The protection from cytotoxicity involved a decrease in the deposition of C3 on the cell, consistent with the effect of DAF on the C3 convertase. A second pair of anchor variants, MCP and a GPI-anchored construct, MCP-PI, were also equivalent in their complement protection.

The equivalent function of GPI-anchored and TM versions of a protein was not expected based on the hypothesized increased lateral mobility of GPI-anchored proteins, which should confer a functional advantage in contacting ligand, in this case, C3b or C4b, on the cell surface. These data suggest either that GPI-anchored and TM versions of a protein have equal lateral mobility in the membrane, or else that increased lateral mobility is not advantageous to DAF or MCP in carrying out their complement inhibitory roles. Furthermore, DAF and MCP demonstrated approximately equal protection of cells from complement-mediated cytotoxicity, suggesting that DAF and MCP provide overlapping levels of protection to cells against damage mediated by the complement system.

T issues are subject to inflammatory damage mediated by the complement (C) system. To keep this pathway in check, multiple control proteins downregulate the action of C. The largest group of control proteins is the regulators of complement activation (RCA)[1] multigene family on the long arm of chromosome 1 (1), including the membrane proteins C3b receptor (CR1), C3d receptor (CR2), decay-accelerating factor (DAF), and membrane cofactor protein (MCP), as well as the serum proteins factor H and C4-binding protein, all of which function at the critical C3 convertase step. CR1 and CR2 have limited distribution on hematopoietic

cells (2), and CR1 appears to function mainly extrinsically to the cell where it resides (3), so these proteins do not appear to be the major elements protecting individual cells from C. On the other hand, DAF and MCP (reviewed in reference 4) are widely distributed on hematopoietic cells and on endothelial and epithelial tissues (5–10), and DAF functions intrinsically to protect cells from complement (11), suggesting that DAF and MCP might be the most important general elements of the RCA multigene family for protecting cells from complement-mediated damage. Another set of regulatory proteins, homologous restriction factor (HRF) (also called C8-binding factor) (12, 13) and membrane inhibitor of reactive lysis (MIRL) (also called HRF20) (14–17), both inhibit at the C8/C9 stage of the membrane attack complex.

Interestingly, DAF, HRF, and MIRL are all glycosyl-phosphatidylinositol (GPI)-anchored membrane proteins (15,

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[1] *Abbreviations used in this paper:* DAF, decay-accelerating factor; GPI, glycosyl-phosphatidylinositol; HRF, homologous restriction factor; MCP, membrane cofactor protein; MIRL, membrane inhibitor of reactive lysis; RCA, regulators of complement activation; TM, transmembrane.

17–23). One of the proposed properties of GPI-anchored membrane proteins is an increased lateral mobility compared with transmembrane (TM) polypeptide-anchored membrane proteins. Specific measurements for DAF (24) and Thy-1 (25) have shown these two GPI-anchored proteins to have lateral diffusion constants more in the typical higher range of membrane lipids than of TM proteins. It has been hypothesized that the GPI anchor permits faster mobility in the membrane and produces an advantage to the protein in carrying out its function. In the case of the GPI-anchored C proteins, the proposed faster lateral mobility would permit more rapid contact of the regulatory protein with the C components to which it is targeted, i.e., DAF to C3b/C4b, and HRF and MIRL to C8/C9. There is no direct evidence, however, for this proposal.

In the present study, we demonstrate directly that GPI-anchored and TM versions of DAF or MCP protect cells equally well from C3 deposition and cytotoxicity. To accomplish this we have established transfected Chinese hamster ovary (CHO) cell lines expressing DAF or MCP, and shown that the transfected human gene products protect foreign cells from human complement.

Materials and Methods

Antibodies. A rabbit polyclonal antibody to human DAF was prepared as described (26). F(ab')₂ was prepared from this antibody by digestion with immobilized pepsin (27) followed by a protein A-Sepharose column to remove F$_c$ fragments and any undigested IgG, according to manufacturer's directions (Pierce Chemical Co., Rockford, IL). The murine mAbs to human DAF IA10 and IIH6 (5) were generously provided by T. Kinoshita (Osaka University, Osaka, Japan), and the mAb 1C6 (28) to DAF was a gift from T. Fujita (Fukushima Medical College, Fukushima, Japan). Murine mAb E4.3 (29) to human MCP was a gift from D. Purcell and I. McKenzie (University of Melbourne, Melbourne, Australia). Murine mAb to human C3c was purchased from Quidel (San Diego, CA), and isotype control (IgG1) mAb was purchased from Accurate Chem. & Sci. Corp. (Westbury, NY). Rabbit polyclonal antibody to hamster PBL (which crossreacts with CHO cells) was obtained from Accurate Chem. & Sci. Corp. (Westbury, NY), and another rabbit polyclonal antibody was produced by immunization with CHO cells.

DNA. We have reported full-length cDNA for DAF (30, 31) and MCP (32). cDNA constructs representing a TM version of DAF and a GPI-anchored version of MCP were made by exchanging the regions of the cDNAs encoding the COOH-terminal domains of each protein using standard techniques of restriction enzyme digestion, isolation of DNA fragments from agarose gels, and subsequent ligation (33). Specifically, DAF-TM consists of amino acids 1–304 of DAF and 270–350 of MCP, and MCP-PI consists of amino acids 1–269 of MCP and 307–347 of DAF (numbering starts from the first amino acid of the mature protein without the signal peptide). An alternate construct for a TM version of DAF using the TM and cytoplasmic domains of HLA-B44 has been reported (34). To confirm in-frame ligation, all constructs were sequenced through the region of the splice using double-stranded DNA (35) and Sequenase (36). The expression vector SFFV.neo (37), which contains the spleen focus-forming virus 5' long terminal repeat, SV40 splicing and polyadenylation signals, and the neomycin-resistance

gene, was a gift from S. Fine and D. Loh (Washington University, St. Louis, MO).

Expression of cDNA. Full-length cDNA for expression was subcloned into the EcoRI site of SFFV.neo (using EcoRI linkers where necessary). This DNA was transfected into CHO cells by lipid-mediated DNA transfection with 10 µg DNA and 100 µg Lipofectin (38). CHO cells were maintained in Ham's F12 medium, 5% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin in an atmosphere of humidified 5% CO₂, 95% air at 37°C. Positive cells were selected by resistance to the neomycin analogue G418 (0.25 mg/ml active drug). These selected cells were then subjected to a variable number of rounds of sterile sorting by flow cytometry (Epics V, Coulter Immunology, Hialeah, FL) using either rabbit polyclonal antibody to DAF or murine mAb E4.3 to MCP as primary antibodies and appropriate FITC-labeled second antibody. Individual subclones were produced by limiting dilution.

Biosynthetic Labeling and Immunoprecipitation. Transfected CHO cells were biosynthetically labeled with [^{35}S]cysteine (>1,000 Ci/mmol). Cells at ∼70% confluence in 100-mm tissue culture plates were washed and placed in cysteine-free medium (cysteine-free Ham's F12 medium, 5% dialyzed FCS, 2 mM glutamine, antibiotics) for 1 h, after which [^{35}S]cysteine was added to 100 µCi/ml, and incubation continued for 4 h. After labeling, the supernatant was removed, cells were washed twice at 4°C with Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4), and then the cells were solubilized in a 1% solution of Triton X-114 in Tris-buffered saline in the presence of the protease inhibitors aprotinin (100 U/ml), PMSF (2 mM), and pepstatin (1 µM). The nonionic detergent Triton X-114 has been used to isolate membrane proteins, for it has the useful property that above the cloud point temperature of ∼20°C, the solution separates into detergent-rich and detergent-poor or aqueous phases, with integral membrane proteins partitioning into the detergent phase (39). The Triton X-114 detergent phase of the cell lysate was immunoprecipitated with either anti-DAF or anti-MCP antibodies using *Staphylococcus aureus* Cowan I strain as an immunosorbent as previously reported (40). Samples were analyzed by SDS-PAGE on 9% acrylamide gels under reducing conditions followed by fluorography as described (40).

Assay for GPI Anchor. Cells were labeled as described above, and the solubilized membrane proteins (from the Triton X-114 detergent phase) were treated with 0.2 U PI-PLC from *Bacillus thuringiensis* (a gift from M. Low, Columbia University, New York, NY) or control buffer at 15°C for 16 h, then separated into detergent and aqueous phases, immunoprecipitated, and analyzed by SDS-PAGE. In some cases, membrane proteins were immunoprecipitated before PI-PLC treatment, which was then carried out at 23°C for 4–16 h. A GPI anchor was indicated by the ability of PI-PLC to release the protein from the detergent phase to the aqueous phase (41).

Flow Cytometry. 1–2 × 10⁶ transfected cells were washed and incubated with saturating concentrations of rabbit polyclonal antibody to DAF or murine mAb to MCP for 30 min at 4°C, then washed and stained with appropriate FITC-labeled second antibody for 30 min at 4°C. In some cases, murine mAb to DAF was used. 10⁴ cells were then analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA) flow cytometer to assess surface expression of DAF or MCP. Quantitative comparison of mean channel fluorescence was used to assess relative expression of DAF or MCP, using one DAF or one MCP transfectant as an arbitrary standard. In some cases, an approximate comparison of the DAF and MCP scales was made by performing flow cytometric analysis of the DAF and MCP standards using IA10 mAb for DAF and E4.3 mAb for MCP, respectively. Both of these mAbs are subclass IgG2a, and

the same FITC-labeled anti–mouse IgG was used for both cells, allowing a semi-quantitative comparison of the DAF and MCP expression levels.

^{51}Cr Release Cytotoxicity Assay. CHO cells were plated at 1.6 × 10^4 per well in 96-well trays 1 d before the assay. On the next morning, the cells were labeled with ^{51}Cr by incubation with 2 μCi per well in complete medium for 3 h at 37°C. Wells were then washed, 25 μl rabbit antiserum or dilution was added, and the plates were incubated on ice for 30 min. Then, 100 μl of human C (or dilution) was added, and the plates were incubated at 37°C for 60 min. 100 μl of supernatant was removed from each well and counted for radioactivity. Cells incubated without antibody or C (control) were used to measure spontaneous ^{51}Cr release, and cells incubated with 1% SDS were used to measure complete release. Specific percent ^{51}Cr release (cytotoxicity) was calculated as: (test cpm – control cpm)/(SDS cpm – control cpm). All samples were done in quadruplicate, and the SEM for cytotoxicity was 1–5%.

In some experiments, DAF function was blocked by preincubation with antibodies. Specifically, after ^{51}Cr labeling, the cells were incubated with 25 μl 1C6 DAF mAb, which has been shown to block DAF function (28), or subclass control mAb MOPC21 (murine IgG1 subclass antibodies do not activate C). After incubation on ice for 30 min, the remainder of the cytotoxicity assay was carried out as detailed above. A similar blocking was done with rabbit polyclonal anti-DAF F(ab′)$_2$ or normal rabbit IgG F(ab′)$_2$.

C3 Deposition on Cells. C3 deposition on cells by the action of antibody and C was measured essentially as described (42). Briefly, CHO transfectants were removed from tissue culture plates by brief trypsinization. After washing, the cells were treated with rabbit antibody and human C as in the ^{51}Cr release cytotoxicity assay. At the completion of those incubations, the cells were washed and sequentially incubated with murine mAb to C3c or subclass control MOPC21 and FITC-labeled anti–mouse IgG, then analyzed by flow cytometry.

Results

Creation of CHO Transfectants Expressing DAF as GPI-anchored or Transmembrane Protein. To be able to compare the protein with alternate modes of membrane attachment, we constructed a version of DAF with the COOH-terminal domain replaced by the TM and cytoplasmic domains of MCP. This cDNA construct, designated DAF-TM, and DAF were subcloned into the expression vector SFFV.neo (37), transfected into CHO cells with lipofectin (38), and selected in G418. To demonstrate the mode of membrane attachment of the transfected proteins, the susceptibility to PI-PLC was tested: PI-PLC releases GPI-anchored proteins from the detergent to the aqueous phase of Triton X-114 by removing the di(acyl/alkyl)glycerol moiety that forms the membrane (or detergent) binding domain of the protein. For this assay the cells are biosynthetically labeled with [^{35}S]cysteine, extracted in Triton X-114, treated with PI-PLC or buffer, phase partitioned, immunoprecipitated with antibodies to DAF, and analyzed by SDS-PAGE and fluorography. The transfected DAF in CHO cells repartitions into the aqueous phase of Triton X-114 after PI-PLC treatment (Fig. 1 *A*), whereas DAF-TM is unaffected by PI-PLC treatment in this assay (Fig. 1 *B*), demonstrating the GPI anchoring of DAF and the TM anchoring of DAF-TM.
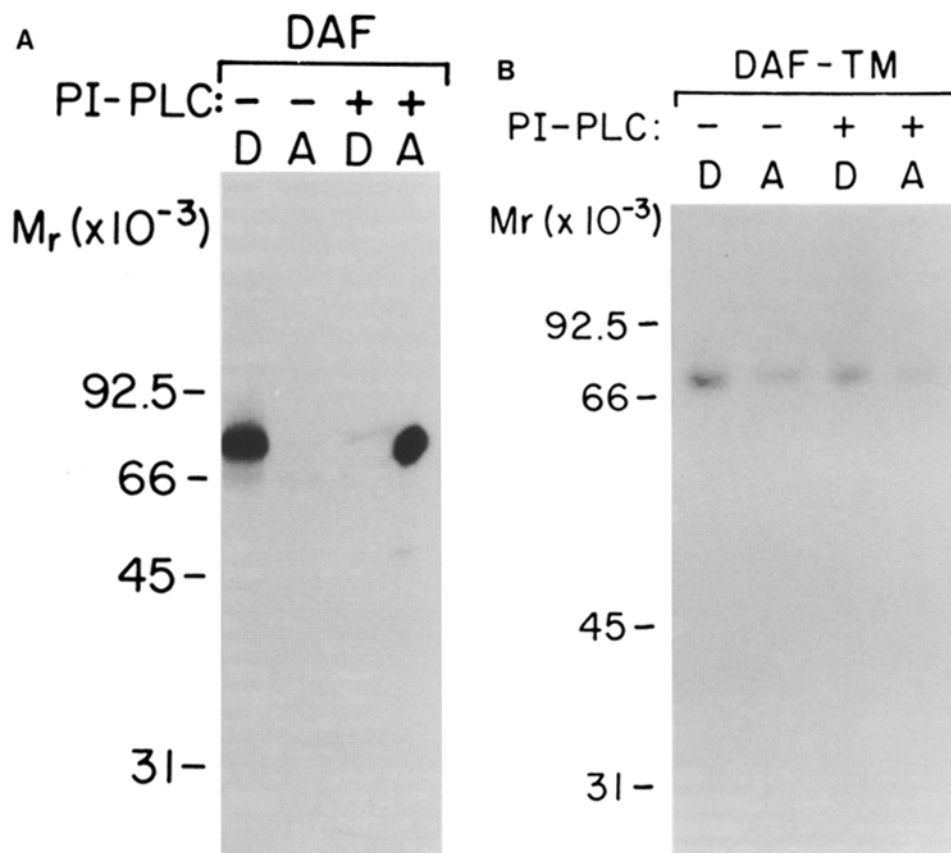

Figure 1. Expression of DAF as a GPI-anchored or TM protein in CHO cells. CHO cells were transfected with the expression vector SFFV.neo containing the cDNA for DAF or the construct DAF-TM, where the COOH terminus of DAF is replaced with the TM and cytoplasmic domains of MCP. Pooled transfectants were selected in the neomycin analog G418. Stable transfectants were assayed for the form of membrane anchor of DAF by biosynthetic labeling with [^{35}S]cysteine, digestion of the Triton X-114 detergent extract with PI-PLC or control buffer, phase separation, immunoprecipitation with anti-DAF antibody, followed by SDS-PAGE and fluorography. The presence (+) or absence (–) of PI-PLC digestion of the sample and the Triton X-114 detergent (*D*) or aqueous (*A*) phase are shown above the lanes. (*A*) DAF; (*B*) DAF-TM.
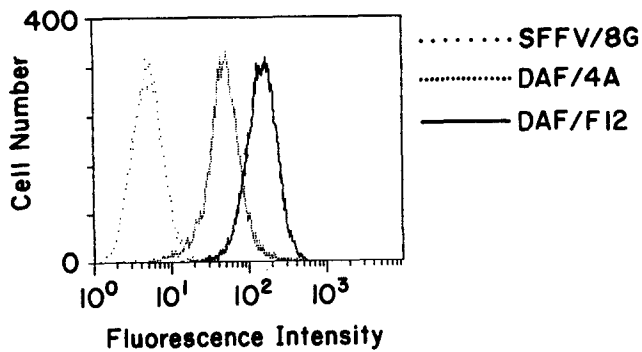
Figure 2. Transfectants that express a range of levels of DAF. Pooled stable transfectants for DAF were sorted for high DAF expression by flow cytometry and cloned by limiting dilution. Individual clones were analyzed by flow cytometry using a rabbit polyclonal antibody to human DAF. Three representative clones are shown in this figure, one from a cell transfected with vector alone (SFFV) to serve as a negative control for immunofluorescence and two individual clones transfected with DAF.
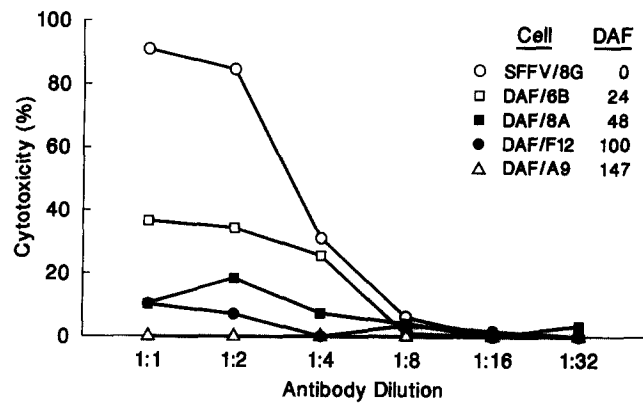
Figure 4. Dose-response effect of transfected DAF in protecting cells from cytotoxicity. A set of DAF clones that express a range of DAF (levels shown in the inset in the figure, with the DAF expression determined by flow cytometry, as in Fig. 2, relative to clone DAF/F12 arbitrarily set to 100 U) was tested in the ^{51}Cr release cytotoxicity assay, as described in the legend to Fig. 3.

We next wanted to create a set of transfectant clones that expressed a range of DAF levels. The pooled transfectants were sorted for high DAF expression by flow cytometry and cloned by limiting dilution. Individual clones were analyzed by flow cytometry and demonstrated a range of expression (two representative DAF clones are shown in Fig. 2).

DAF Protects CHO Transfectants from C-mediated Cytotoxicity. To study the function of the transfected DAF molecule, the CHO transfectants were tested in a ^{51}Cr release cytotoxicity assay that used a rabbit polyclonal antibody (raised against hamster lymphocytes) and human C. Several control CHO transfectants (SFFV vector only) were lysed, whereas a CHO transfectant expressing DAF was almost completely protected from cytotoxicity (Fig. 3 A). Controls involving antibody or C alone showed that both were required for lysis. Inactivation of the human C at 56°C for 30 min to destroy both C pathways abrogated all cytotoxicity, and inactivation of the human C at 50°C for 20 min to destroy the alternate pathway of C demonstrated that this was a classical pathway-mediated event, as expected for an antibody-initiated process (data not shown).

A second rabbit polyclonal antibody that was raised against CHO cells was tested in the cytotoxicity assay, and the DAF

transfectants were also relatively protected from C-mediated cytotoxicity (Fig. 3 B). This antibody had a higher titer than the previous antibody (compare Fig. 3, A and B). The degree of protection, although not as complete as with the previous antibody, was still quite marked (Fig. 3 B), and provides evidence that this finding is not limited to any specific antibody.

Glycophospholipid-anchored and Transmembrane Versions of DAF Protect Cells Equally Well from C-mediated Cytotoxicity. The data (Fig. 3) demonstrate that the CHO cells transfected with DAF are protected from C-mediated cytotoxicity. This provides a system for testing mutations in DAF to assess the effect of the variation on function. Specifically, we address the question of whether the GPI anchor of DAF confers any functional advantage (or disadvantage) over a polypeptide TM anchor. To compare the functional efficiency of the two versions of DAF, we established a set of CHO transfectants expressing a range of levels of DAF or DAF-TM. The relative level of DAF expression on any clone was quantitated by flow cytometry (see Fig. 2 as a representative example) using one DAF clone as a standard, arbitrarily designated as 100 U. The surface expression was assessed with a rabbit polyclonal anti-
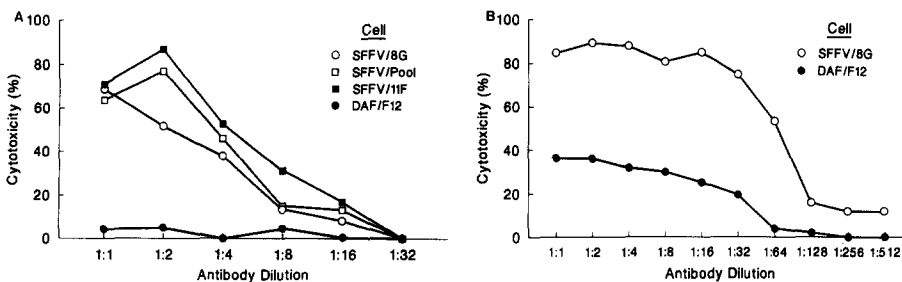

Figure 3. Transfected DAF protects CHO cells from antibody plus C-mediated cytotoxicity. A CHO cell line transfected with DAF was compared with several control cell lines transfected with SFFV vector alone, using a rabbit antibody plus human C ^{51}Cr release cytotoxicity assay, as described in Materials and Methods. (A) The results of the cytotoxicity assay show a titration curve of percent cytotoxicity vs. antibody dilution. The rabbit polyclonal antibody was raised against hamster lymphocytes.

Two clones from CHO cells transfected with vector alone (SFFV) are shown, along with the pool of cells from the SFFV transfection and a clone from the CHO cells transfected with DAF. The assay is done in quadruplicate, and SEM ranges from 1% to 5%. DAF clearly protects the cells from cytotoxicity compared with any of the control transfectants. (B) Cytotoxicity assay comparing one of the DAF and control (SFFV) clones using a rabbit antibody raised against CHO cells, again demonstrating the protective effect of transfected DAF.
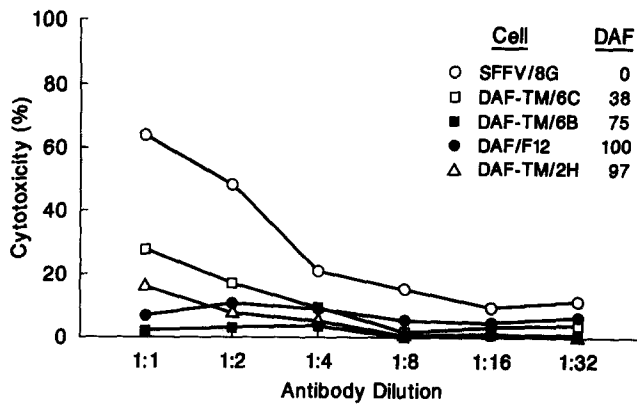
Cell	DAF
○ SFFV/8G	0
□ DAF-TM/6C	38
■ DAF-TM/6B	75
● DAF/F12	100
△ DAF-TM/2H	97

Figure 5. Transfected DAF-TM has an equivalent effect to DAF in protecting cells from C. A set of DAF-TM clones that express the same range of DAF levels as the set of DAF transfectants in Fig. 4 was tested in the cytotoxicity assay, as described in the legend to Fig. 3. A single DAF transfectant clone was also run in this assay for comparison. Although there was a small assay-to-assay variation in the level of cytotoxicity (compare the maximum 91% cytotoxicity of clone SFFV/8G in Fig. 4 with the maximum 64% cytotoxicity of the same clone in Fig. 5), the overall dose-response effect of increasing protection with increasing DAF expression was similar and covered the same quantitative range for the DAF-TM clones as for the DAF clones tested in Fig. 4.

DAF. However, because of concern that the alternative modes of membrane anchoring might affect the binding of antibody to some epitopes, the quantitation was repeated with anti-DAF mAbs IA10 and IIH6 (5), and both mAbs gave the same quantitation as the polyclonal antibody (data not shown).

A set of DAF clones was then tested in the antibody plus C cytotoxicity assay. There is a clear dose-response relationship between the expression of transfected DAF in a clone and the amount of protection from cytotoxicity (Fig. 4). This finding in itself provides additional support for the conclu-
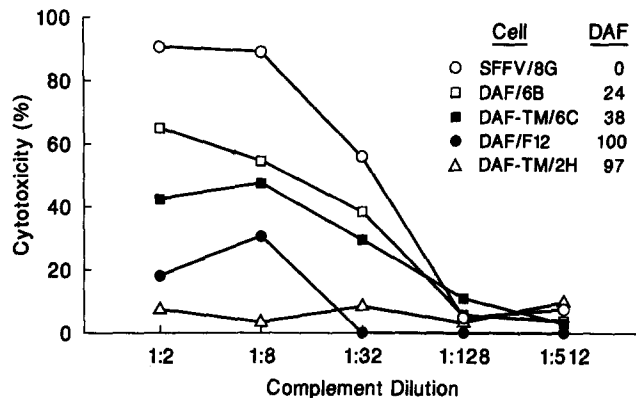

Cell	DAF
○ SFFV/8G	0
□ DAF/6B	24
■ DAF-TM/6C	38
● DAF/F12	100
△ DAF-TM/2H	97

Figure 6. Equivalent cytotoxicity protection of DAF and DAF-TM seen under conditions of limiting C dilution. The previous cytotoxicity assays all used antibody dilutions and full-strength C. To compare the two versions of DAF under conditions of limiting C, the cytotoxicity assay was performed as a C dilution, with all other procedures are as described in the legend to Fig. 3. A low and a high expression DAF/DAF-TM pair were both tested in this cytotoxicity assay, and again demonstrated the equivalent protective effect of DAF and DAF-TM under these conditions of limiting C.
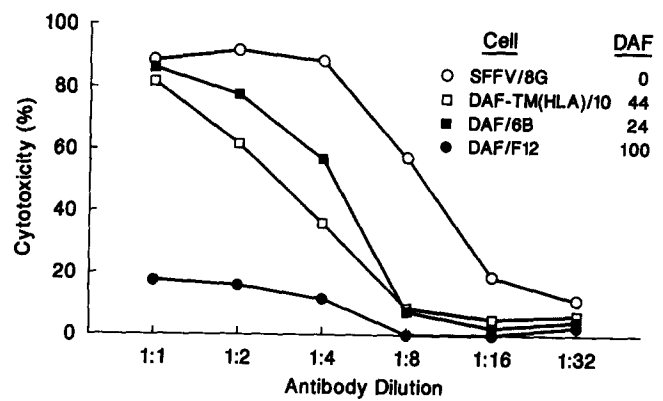

Cell	DAF
○ SFFV/8G	0
□ DAF-TM(HLA)/10	44
■ DAF/6B	24
● DAF/F12	100

Figure 7. An alternate TM version of DAF is also equivalent to GPI-anchored DAF in its C-protective effect. The DAF-TM construct tested above uses the TM and cytoplasmic domains of MCP. An alternate DAF-TM was constructed with the TM and cytoplasmic domains of HLA-B44. This was expressed in CHO as described in the legend to Fig. 1, and tested by the cytotoxicity assay as described in the legend to Fig. 3. A clone expressing the DAF-TM(HLA) construct was compared with clones expressing DAF, and it falls right on the same dose-response curve of cytotoxicity protection as DAF.

sion that the protection from cytotoxicity is specifically caused by the transfected DAF. When a set of DAF-TM clones covering a similar range of DAF expression was surveyed, they showed an equivalent dose-response relationship (Fig. 5). These assays using undiluted C and a range of antibody dilutions represent conditions of limiting antibody. Reasoning that subtle differences in protection might be more apparent under conditions of limiting C, the cytotoxicity assay was also run under these conditions. The same equivalent efficiency of DAF and DAF-TM in protecting the cells from C-mediated cytotoxicity was seen (Fig. 6). Thus, versions of DAF with either a GPI or a TM anchor demonstrate equal efficiency in protecting the cell from C-mediated cytotoxicity.

A second TM version of DAF, which uses the TM and cytoplasmic domains of HLA-B44 (34) instead of MCP, was also tested. A transfectant expressing this alternate DAF-TM was protected from C-mediated cytotoxicity, with the degree of protection falling right on the DAF-response curve (Fig. 7). Therefore, the equivalence of the GPI and TM anchors for DAF in the protection from C-mediated cytotoxicity does not appear to vary with different TM anchors.

Protection from C-mediated Cytotoxicity Conferred by Transfected DAF Involves DAF Functional Domain and Results in Decreased C3 Deposition. All of the data on cytotoxicity involve the end result of C damage to the cell by the membrane attack complex. DAF functions to regulate the C cascade by its inhibitory action on the C3 convertase (43), which then has the downstream effect of decreasing the overall level of cytotoxicity. The next set of experiments were designed to confirm that the protective effect seen in DAF transfectants does in fact involve the functional domain of DAF. The first approach was to block the DAF functional site with antibodies and demonstrate reversal of the protection. Specifically, the transfectants were preincubated with blocking antibodies before addition of the rabbit antibody and human C. Pretreat-
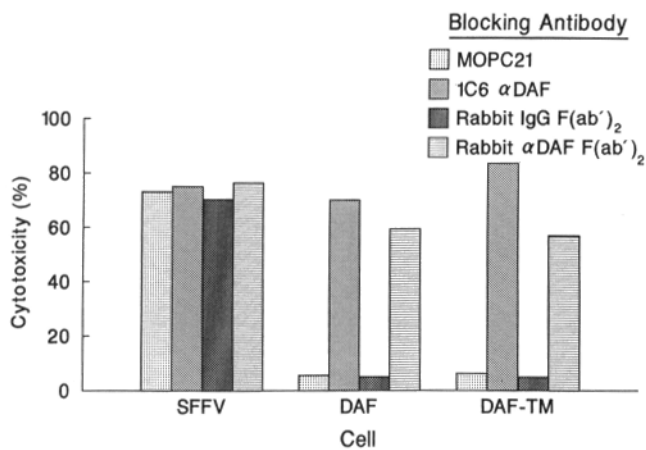
Figure 8. Blocking DAF functional domain with antibody reverses C-protective effect of transfected DAF and DAF-TM. A vector control–transfected clone (SFFV/8G), clone DAF/F12, and clone DAF-TM/2H (the latter two clones expressing equal amounts of surface DAF) were tested in the ^{51}Cr release cytotoxicity assay, as described in the legend to Fig. 3, with the following addition: the cells were pretreated with anti-DAF blocking antibody or isotype control, as indicated in the graph inset, for 30 min at 4°C before being incubated with rabbit antibody and human C. Blocking with either mAb 1C6 or F(ab')₂ rabbit anti-DAF reverses the protection from cytotoxicity afforded by the transfected DAF with either GPI or TM anchor.

Figure 9. Protection from cytotoxicity of cells transfected with DAF or DAF-TM is marked by reduced C3 deposition. The clones SFFV/8G, DAF/F12, and DAF-TM/2H (same clones as used in Fig. 8) were briefly trypsinized from plates, then treated exactly as in the cytotoxicity assay outlined in the legend to Fig. 3, except there was no ^{51}Cr labeling step. After the rabbit antibody and human C incubation steps, the cells were washed and treated sequentially with mouse mAb against human C3c or isotype control MOPC21 followed by FITC-labeled goat anti–mouse IgG, and then analyzed for fluorescence by flow cytometry. SFFV cells treated with MOPC21 give the background immunofluorescence.

ment of the DAF or DAF-TM transfectants with DAF mAb 1C6, which blocks DAF function (28), led to reversal of the protective effect of the transfected DAF (Fig. 8). Control mAb MOPC21 of the same subclass had no effect, and the mAbs had no effect on the SFFV (vector only control) transfectant. Similarly, rabbit polyclonal anti-DAF F(ab')₂, but not control rabbit IgG F(ab')₂, also reversed the protective effect of the transfected DAF (Fig. 8).

Next, to show that the transfected DAF is actually having an effect on C3 deposition, we measured C3 levels on the cells. Transfectants were treated with rabbit antibody and human C as in the cytotoxicity protocol (omitting the ^{51}Cr labeling), and they were then analyzed by flow cytometry for surface C3 expression using a mAb against C3c. The DAF and DAF-TM clones tested had equal DAF expression, and this resulted in equal reductions in C3 deposition compared with the control SFFV transfectant (Fig. 9). Thus, the protective effect of the transfected DAF is specific for the DAF functional domain and results in decreased C3 deposition.

GPI-anchored and Transmembrane Versions of MCP Show Equivalent Protective Effect against C-mediated Cytotoxicity. To generalize the present finding that DAF functions equivalently in inhibiting the C cascade whether anchored by a GPI or TM structure, we tested another pair of anchor variants. The purified membrane protein MCP has cofactor activity for the factor I–mediated proteolysis of C3b and C4b when assayed in the fluid phase (44), and it is presumed that the intrinsic membrane protein has similar activity and thus protects the cell from C damage. MCP is a TM protein; we constructed a variant of MCP that is GPI anchored by replacing the region of the MCP cDNA that encodes the TM and cy-
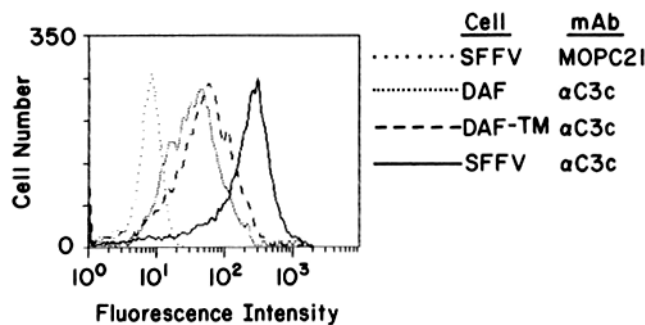
toplasmic domains with the region of the DAF cDNA that encodes the COOH-terminal signal for GPI anchor addition. Testing of CHO transfectants expressing these two versions of MCP, the wild-type MCP and MCP-PI, with PI-PLC established that they do in fact encode membrane proteins that use TM and GPI anchors, respectively (Fig. 10).

Cell sorting followed by limiting dilution cloning was used to produce clones with a range of surface expression of MCP, just as reported above for DAF. Transfectants expressing approximately equal amounts of MCP or MCP-PI were tested in the antibody plus C cytotoxicity assay (Fig. 11). This demonstrated, first, that MCP does act as an intrinsic C-protective molecule in the cell membrane, and, second, that it carries out this function equivalently with either a TM or GPI anchor.

Finally, these results provide a comparison of the C-protective effect of DAF vs. MCP. Note that the level of expression of MCP is determined by flow cytometry with E4.3 mAb, with the units of expression relative to one arbitrarily selected clone. However, a reasonably quantitative comparison can be made between the DAF and MCP transfectants by using a mAb of the same subclass for each protein together with the same FITC-labeled goat anti–mouse IgG second antibody. This was done using the DAF mAb IA10 (5) and the MCP mAb E4.3 (29), both of the murine IgG2a subclass, and it showed that the MCP and MCP-PI clones in Fig. 11 had approximately half as many surface molecules of the transfected cDNA as the DAF clone. Although these comparisons are only semi-quantitative, the cytotoxicity assay therefore shows that the MCP and MCP-PI transfectants had the same approximate level of protection from C-mediated cytotoxicity as equivalent DAF transfectants (compare Fig. 4 with Fig. 11). Thus, these data not only establish that MCP functions as an intrinsic membrane protein to protect against C-mediated cytotoxicity with either a TM or GPI anchor, but it suggests that DAF and MCP are similar in their efficiency for carrying out this protection of cells.
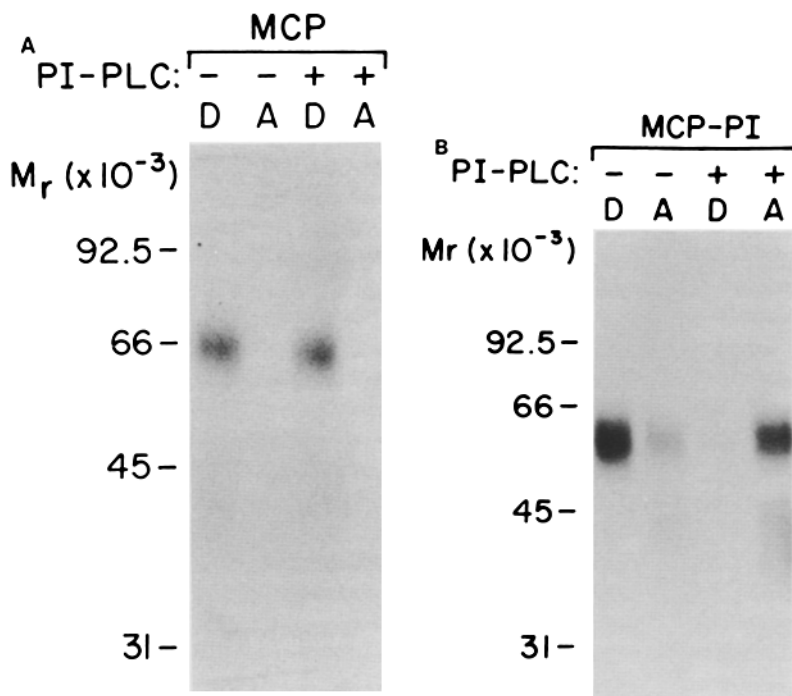
Figure 10. Expression of MCP as a TM or GPI-anchored protein in CHO cells. The cDNAs for MCP or a construct designated MCP-PI where the TM and cytoplasmic domains of MCP are replaced with the COOH-terminal domain of DAF (which encodes the signal for GPI anchor attachment) were expressed in CHO cells and tested for the mode of membrane anchoring with PI-PLC. Protocols and figure labels are described in the legend to Fig. 1. (*A*) MCP; (*B*) MCP-PI.

Discussion

The two main results of this study are the demonstration that transfected foreign cells expressing human DAF or MCP are protected from C-mediated cytotoxicity, and the finding that both GPI-anchored as well as TM versions of these proteins function equally well in carrying out this C protection. The former result confirms the known role of DAF on the cell and establishes that MCP can also carry out this C-protective effect intrinsically on a cell. The latter finding goes against a hypothesized role for the GPI anchor on a mem-

Figure 11. Transfected MCP or MCP-PI protect cells equivalently from C-mediated cytotoxicity. Clones expressing MCP or a GPI-anchored version of MCP, MCP-PI, at approximately equal levels were tested in the cytotoxicity assay, as described in the legend to Fig. 3, along with an SFFV vector control and a DAF transfectant.

brane protein, leaving open the question of what, if any, advantage is conveyed by having a GPI anchor on DAF.

The study of the physiological role of membrane DAF and MCP has been approached at three increasingly specific levels: (*a*) functional analysis of the purified protein extracted from the membrane; (*b*) study of the protein in the membrane using antibodies to block function; and (*c*) in the case of DAF, reincorporation of the purified protein into the cell membrane. These proteins in solution could inhibit the C3 convertases of either the classical or alternate pathways, DAF by inhibiting the formation and accelerating the decay of C3 convertases (43), and MCP by acting as a cofactor for the factor I–mediated proteolytic cleavage of C3b or C4b (44). That DAF could carry out this function when present in the membrane of an intact cell was shown by blocking the DAF function with antibody (45): a cell that naturally expresses DAF could be compared with the same cell without DAF (DAF function blocked by antibody). Three membrane proteins are known to block C3 convertase function, CR1, DAF, and MCP. CR1, with a limited tissue distribution, functions extrinsically on C3 convertases in the fluid phase or on other cells and does not appear mainly to serve the role of protecting the individual cell where it is located (3). DAF does function intrinsically (11). The function of MCP on intact cells has not been explored until recently, although MCP has been hypothesized to function similarly to DAF in this regard. The antibody blocking data on DAF was done on human erythrocytes, which do not possess MCP. There were no cells or cell lines known that possess MCP but not DAF or CR1, so similar data on the function of MCP were not available. While this manuscript was in preparation, a report by Seya et al. (46) appeared that identified two such human T cell lines, and studies using MCP mAb blocking demonstrated that MCP was functioning
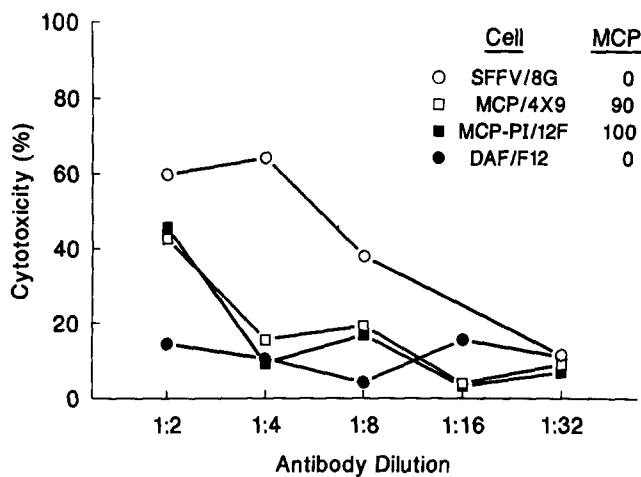
on the cells to block C. The final and most direct approach to the function of these molecules involves incorporation into a cell that does not possess them; and for DAF, this was made possible because of this GPI anchor: the purified molecule would reincorporate into cells as an integral membrane protein (11). This made possible direct studies of cells with and without DAF and confirmed the function of the molecule in the plasma membrane (11). This was not possible for TM molecules such as MCP.

The present model based on transfected cells extends the ability to study the function of these proteins, essentially without restriction. The cloned cDNA is expressed by transfection in CHO cells, and then these cells can be tested for protection from the action of human C. We have shown that an assay based on antibody plus C functions in this system. This can involve the overall effect of cytotoxicity (Fig. 3) or a more specific point in the C pathway such as C3 deposition (Fig. 9). We have used this system to demonstrate that both DAF and MCP can protect cells from C (Figs. 4 and 11). This confirms the results involving DAF reincorporation into cell membranes (11) and provides the most direct evidence that MCP functions in a cell membrane as a C-protective molecule. This model is particularly valuable because of the ability to study any variant of a molecule. In this report, we made use of this approach to study variants of DAF and MCP that used alternate membrane anchoring (DAF-TM and MCP-PI). The same methodology can be used to study point mutations or deletions in the cDNA to map structural or functional domains in the encoded protein (Lublin, D.M., manuscript in preparation).

In this study, we found that DAF and MCP had roughly equivalent C-protective effects. This conclusion is based on semi-quantitative comparisons of the levels of surface expression of DAF and MCP by flow cytometry, and it is not to be interpreted beyond an order-of-magnitude comparison; a more precise comparison will require quantitative measurements by immunoassay. Another important question for investigation is whether DAF and MCP, using different mechanisms for inhibiting the C3 convertase, can act synergistically, or do they simply provide a level of redundancy in protecting cells.

The finding that both DAF and MCP can be incorporated into cells and protect them from C has implications for the possible therapeutic use of these C regulatory proteins as immunomodulators. Two major points in the C cascade where regulatory proteins function is at the C3 convertase stage (RCA family) and at the membrane attack complex stage (HRF and MIRL). Although the latter group might have a greater direct effect on cytotoxicity, the RCA proteins not only block cytotoxicity but also block the deposition of C3b/C4b on the cell (which makes it a target for clearance by CR1-bearing phagocytic cells) and the release of the anaphylatoxic and chemotactic C peptides C3a and C5a. In a recent report, a soluble form of recombinant human CR1 was shown to have antiinflammatory properties in a rat model of reperfusion in-

jury (47). Soluble forms of DAF and MCP should function similarly as therapeutic agents in autoimmune and other inflammatory states; CR1 has the advantage of possessing both decay-accelerating activity and cofactor activity, but DAF and MCP together have both C3 convertase inhibitory functions and might offer an advantage due to their three times smaller M_r. However, a potentially much greater advantage stems from the GPI anchor of DAF. Purified DAF can be reincorporated into cells (11), and this cellular form has markedly higher activity in inhibiting the C3 convertase than a soluble form of DAF (18, 19). Thus, one potential approach would be to treat tissues directly with DAF and allow incorporation in the cell membranes. There are many potential technical difficulties, though one area where it might be easily applicable is transplantation. Because we have also constructed a GPI-anchored form of MCP and shown in this study that it can protect cells from C, both recombinant DAF and MCP-PI could be used in this manner.

One of the original motivations for this study was to test the hypothesis that attachment of a protein to the plasma membrane by a GPI anchor instead of a TM region and cytoplasmic tail would be advantageous to function. Direct measurements of lateral diffusion constants for DAF (24) and Thy-1 (25) indicated a high lateral mobility for these two GPI-anchored membrane proteins. This led to the suggestion that GPI-anchored proteins would have higher lateral mobility than TM proteins, and because of the presumed advantage of lateral mobility, e.g., in contacting a ligand on the cell or in the extracellular medium or on another surface, the above hypothesis was advanced. This hypothesis concerning the functional advantage of a GPI anchor due to increased lateral mobility has been widely cited in reviews (48, 49), yet there has been no evidence to support (or refute) it. The present study has shown that GPI and TM versions of DAF or MCP function equally well in protecting cells from C3 deposition and cytotoxicity. An alternative DAF-TM construct using a different TM and cytoplasmic region was also tested and found to match GPI-anchored DAF in C protection, so this finding is not a function of a particular TM sequence. There are two possible ways to interpret this conclusion: either the putative greater lateral mobility of a GPI-anchored vs. TM protein does not impart a functional advantage, or else the two versions of the protein have equivalent lateral mobility. The latter is really the crux of the matter, for the reports to date simply measure the lateral mobility of a GPI-anchored protein and by comparison with the whole range for TM proteins or lipids, suggest that the GPI anchor allows faster lateral mobility. Clearly, the definitive measurement is to compare GPI and TM versions of the same protein. It is logical (but unproven) that faster movement would be advantageous to DAF in contacting C3 convertases. The present results are in fact a functional measurement suggesting equal lateral mobility for GPI and TM versions of a protein. However, final interpretation must await direct measurements of the lateral mobility of protein pairs such as DAF and DAF-TM.

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