Generation of a Recombinant, Membrane-targeted Form of the Complement Regulator CD59

ACTIVITY IN VITRO AND IN VIVO*

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Inappropriate activation of complement contributes to pathology in diverse inflammatory diseases. Soluble recombinant forms of the natural cell membrane regulators of complement are effective in animal models and some human diseases. However, their use is limited for reasons related to cost, short half lives, and propensity to cause unwanted systemic effects. Some of these limitations may be overcome by use of bacterial expression systems, specific targeting moieties, and judicious choice of regulator. Here we describe the application of these strategies to the generation of a membrane-targeted form of CD59. A recombinant soluble form of rat CD59, comprising the first 71 residues of the mature protein and missing the membrane-anchoring signal, was expressed in bacteria, purified, and refolded in a fully active form. The protein was coupled through its carboxyl terminus to a short, synthetic address tag that confers membrane binding activity. Attachment of the membrane address tag markedly increased complement-inhibitory activity assessed in vitro in hemolysis assays. Intra-articular administration of the tagged membrane regulator of MAC assembly (10). Recombinant soluble forms of CD59 (sCD59) have been generated with the aim of developing a specific inhibitor of MAC for therapy (11–13). However, sCD59 is a poor inhibitor of MAC both in vitro and in vivo. In vitro, sCD59 inhibits efficiently in “reactive lysis” systems where purified components are used to assemble the MAC on targets, but this inhibitory activity is markedly reduced in the presence of serum (11, 14). In vivo, the problem is compounded by the small size of sCD59, which permits rapid clearance in the kidney. Attempts have been made to generate more active forms of sCD59 by designing chimeras or fusion proteins (15, 16), but no clear evidence of MAC-inhibiting activity in vivo has yet been reported. We have taken a different approach to the problem of targeting and retaining C therapeutics by utilizing a membrane-associating “tag” that can be coupled to proteins. Several C regulators have previously been modified in this manner, particularly a small molecule derived from human sCR1 and a portion of the rat C regulator Crry (17, 18). In each case, the addition of the membrane tag markedly increased the regulatory capacity of the parent molecule (10). Recombinant soluble forms of CD59 (sCD59) have been generated with the aim of developing a specific inhibitor of MAC for therapy (11–13). However, sCD59 is a poor inhibitor of MAC both in vitro and in vivo. In vitro, sCD59 inhibits efficiently in “reactive lysis” systems where purified components are used to assemble the MAC on targets, but this inhibitory activity is markedly reduced in the presence of serum (11, 14). In vivo, the problem is compounded by the small size of sCD59, which permits rapid clearance in the kidney. Attempts have been made to generate more active forms of sCD59 by designing chimeras or fusion proteins (15, 16), but no clear evidence of MAC-inhibiting activity in vivo has yet been reported. We have taken a different approach to the problem of targeting and retaining C therapeutics by utilizing a membrane-associating “tag” that can be coupled to proteins. Several C regulators have previously been modified in this manner, particularly a small molecule derived from human sCR1 and a portion of the rat C regulator Crry (17, 18). In each case, the addition of the membrane tag markedly increased the regulatory capacity of the parent molecule in vitro and, in the case of the sCR1 fragment, also markedly enhanced activity in animal models. The targeting technology has been coupled with development of bacterial expression systems for these complex proteins that enable large amounts of active protein to be generated economically.

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‡ The abbreviations used are: C, complement; MAC, membrane attack complex; GPE, guinea pig erythrocyte; AIA, antigen-induced arthritis; sCD59, soluble CD59; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; mAb, monoclonal antibody; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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CD59 inhibits MAC assembly by incorporating tightly into the forming complex (10, 19). The molecule is held together by four disulfide bonds that maintain a rigid, compact, discoid structure. Soluble recombinant forms of CD59 have been expressed in mammalian cells, insect cells, and yeast and shown to be functional in “reactive lysis” assays in vitro (11, 20, 21). Despite its small size, expression of CD59 in a bacterial system has not previously been reported and represents a considerable challenge here to the complex disulfide bridging and tertiary structure. Here we describe the production in Escherichia coli of a recombinant soluble form of rat CD59, modified such that an additional Cys residue is available at the carboxyl terminus. This molecule was fully active as an inhibitor of MAC assembly when compared with soluble rat CD59 expressed in mammalian cells.

The addition of a membrane address tag at the carboxyl terminus generated a membrane binding molecule that had markedly increased MAC-inhibitory activity in vitro when compared with the untagged molecule. Intra-articular administration of the membrane-tagged rat CD59 in rats efficiently inhibited disease progression and joint destruction in a rat arthritis model. These data demonstrate for the first time that specific inhibition of MAC can inhibit pathology in a C- mediated disease model.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

Chemicals and reagents were obtained from Fisher or Sigma unless otherwise stated. pET26b and E. coli BL21(DE3) were obtained from Novagen (Darmstadt, Germany). Restriction enzymes and T4 DNA ligase were purchased from Invitrogen. Oligonucleotide primers were either purchased from Invitrogen or synthesized in house using an Applied Biosystems 392 RNA/DNA synthesizer. DNA sequencing was carried out in house on an Applied Biosystems 373A DNA sequencer utilizing the ABI Prism dye terminator kit. PBS is 8.1 mM Na2PO4, 1.5 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4. CFD is 2.8 mM barbituric acid, 145.5 mM NaCl, 0.8 mM MgCl2, 0.8 mM CaCl2, 0.9 mM sodium barbital, pH 7.2 (Oxoid Ltd., Basingstoke, UK). NZCYM medium contained 1% bactotryptone, 0.5% bacto yeast extract, 0.5% NaCl, sodium barbital, pH 7.2 (Oxoid Ltd., Basingstoke, UK). NZCYM medium contained 1% bactotryptone, 0.5% bacto yeast extract, 0.5% NaCl, sodium barbital, pH 7.2.

Normal human serum was obtained by venepuncture from healthy volunteers. Normal rat serum and guinea pig erythrocytes were obtained from the local animal facility. Monoclonal mouse anti-rat CD59, a cDNA encoding a soluble recombinant form of rat CD59 (sCD59), was engineered and amplified (sCD59-Cys). The primers used contained from the local animal facility. Monoclonal mouse anti-rat CD59, a cDNA encoding a soluble recombinant form of rat CD59 (sCD59), was engineered and amplified (sCD59-Cys). The primers used

Construction of Soluble Recombinant Rat CD59 cDNA Bacterial Expression Vector

Using as template a plasmid containing the full coding sequence for rat CD59, a cDNA encoding a soluble recombinant form of rat CD59 (residues 1–71 of the mature protein sequence, missing the glycosylphosphatidylinositol anchor addition sequence) and including at the carboxyl terminus a 7-residue spacer (GGGGSGGS) and a terminal cysteine, was engineered and amplified (sCD59-Cys). The primers used were as follows: 1) 5’-GGTCCCAACAGGTATCTATCTCGATGC-3’, which included a restriction site (SalI) immediately up-stream of the nucleotides encoding the initiating methionine of the recombinant protein; 2) 5’-GGGCGATCCCTTTAGACGAGACCCGCGCGATCCCTTTAGACGAGACCCGCGCGAAGGACCACGGATTGGTTACAACATGGCCGGC-3’, which included the spacer and terminal Cys upstream of a stop codon (italic type) and a BamHI restriction site (Boldface type). The PCR product (286 bp) was subcloned into the plasmid TOPO-TA® (Invitrogen) and sequenced to confirm fidelity. The plasmid was then digested with NdeI and BamHI to release the insert, which was purified and ligated into the bacterial expression vector pET26b (Invitrogen) digested at the corresponding sites.

Bacterial Expression of sCD59-Cys by Fermentation

The sCD59-Cys cDNA in pET26b was transformed into electrocompetent E. coli BL21 (DE3) bacteria (Novagen, Nottingham, UK) by electroporation (GenePulser™, Bio-Rad). Cells were then plated onto LB agar containing kanamycin (50 μg/ml) to select for clones containing the plasmid. Positive colonies were picked and expanded, and the presence of insert was confirmed by PCR screening. A single positive colony was picked into 25 ml of LB broth containing kanamycin (50 μg/ml) and grown overnight at 37 °C in a shaking incubator. A 20-ml aliquot of this starter culture was inoculated into 2 liters of NZCYM medium containing kanamycin (50 μg/ml) in a Bioflo 3000 Bioreactor (New Brunswick Scientific, Edison, NJ) with a 2-liter bioreactor culture vessel. The fermenter was prepared and run as described previously (18). Cultures were grown for 4 h until the bacteria were in their log phase of growth (A600 = 5–8). Protein expression was then induced by adding sterile filtered isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM.

Molecular weight markers are shown on the left. The arrow indicates the anticipated position of the sCD59-Cys protein.

Isolation, Solubilization, and Refolding of sCD59-Cys from Bacterial Inclusion Bodies

The cell pellet obtained above was thawed, resuspended in lysis buffer (50 mM Tris, 1 mM EDTA, 50 mM NaCl, pH 8.0, 0.5 μg/ml of pellet), and disrupted by two passes at pressure >12,000 p.s.i. through an Emulsiflex CES High Pressure Homogenizer (Glen Creston, Stanmore, UK) at 4 °C. The homogenate was immediately centrifuged (10,000 × g, 10 min, 4 °C), and the pellet containing sCD59-Cys in inclusion bodies was washed three times by centrifugation in 50 mM Tris, 1 mM EDTA, 2% sodium deoxycholate (w/v), pH 8.0, before being resuspended to ~5 mg/ml in solubilization buffer (8% urea, 20 mM Tris, 1 mM EDTA, 50 mM 2-mercaptoethanol, pH 8.5).

Conditions for refolding of sCD59-Cys were chosen empirically by testing a large panel of solutions differing in their pH, presence of reducing and denaturing agents, presence of detergents, etc. as previously described (18, 24). Successful refolding was initially assessed by demonstrating the acquisition of epitopes recognized by the conformation-dependent mAb 6D1 in dot blots. In the chosen protocol, sCD59-Cys was refolded from solubilized inclusion bodies by rapid dilution (1:40) into 50 mM Tris, 1 mM NaCl, 1 mM reduced glutathione, 3 mM oxidized glutathione, pH 8.0. The refold mixture was incubated for 1 h at 4 °C and then buffer-exchanged into PBS and concentrated 10-fold by ultrafiltration (Amicon, Inc., Beverly, MA). Aggregated and/or misfolded protein was removed by ammonium sulfate precipitation. Protein in PBS was diluted 1:2.5 into 3.8 mM ammonium sulfate, 0.1 mM sodium phosphate buffer, pH 6.5, incubated for 10 min at room temperature, and centrifuged. The superna-
sulfate, 0.1 M sodium phosphate buffer, pH 6.5. Precipitated material was separated from the supernatant by centrifugation. Samples of the reader at 410 nM, and percentage of hemolysis at each serum dilution sorbance was read in a Bio-Rad enzyme-linked immunosorbent assay controls contained 1% Triton in place of serum. Plates were spun, and

Modification of sCD59-Cys with a Membrane Address Tag

A membrane address tag was added to sCD59-Cys by derivatization at the free carboxyl-terminal cysteine with the sulfhydryl-reactive peptide, N-myristoylGSSKSPKKKKKPGDC-(S-2-thiopyridyl) C-amide (termed APT542; Adprotech Ltd.) (25), to produce sCD59-APT542. The tagged protein was separated from untagged and purified to homogeneity by cation exchange chromatography on a Mono-S (Amer-

SDS-PAGE and Immunoblotting

Samples were resolved by SDS-PAGE using 4–12% Bis-Tris gradient gels (Novex) according to the manufacturer’s instructions. Protein bands were visualized by staining with Coomassie Blue R-250. For Western blotting, proteins were transferred to nitrocellulose mem-

Hemolysis Assays

Two types of hemolysis assay were used to assess the function of sCD59-Cys and sCD59-APT542.

Nonwash Assays—Guinea pig E (GPE) (2% in CFD) were incubated (15 min, 37 °C) with C8-depleted human serum diluted 1:5 in CFD in order to generate C5b-7 sites on the cells (GPE5b-7). The GPE5b-7 were washed and resuspended to 2% in PBS plus 10 mM EDTA, and 100-μl aliquots were delivered to wells of a 96-well round-bottomed plate. Dilutions of rat serum in PBS plus 10 mM EDTA (100 μl) were then added to individual wells as a source of C8 and C9 and incubated for 30 min at 37 °C. Zero lysis controls contained no rat serum, and 100% controls contained 1% Triton in place of serum. Plates were spun, and supernatant was removed to a fresh 96-well flat-bottomed plate. Absorbance was read in a Bio-Rad enzyme-linked immunosorbent assay reader at 410 nm, and percentage of hemolysis at each serum dilution was calculated relative to the zero and 100% controls. From this titra-

Induction and Treatment of Antigen-induced Arthritis (AIA)

Male Lewis rats (approximately 200 g) were obtained from Charles River (Margate, UK) and housed at Biomedical Services (University of Wales College of Medicine, Cardiff). Rats were allowed free access to food and water and kept in light/dark cycles of 12 h. AIA was induced following an established protocol (17). Briefly, animals were injected subcutaneously with an emulsion of equal volumes of methylated bo-

For assessment of therapeutic effects, AIA was induced in groups of six animals, groups receiving either sCD59-Cys (250 μg in 0.1 ml of PBS), sCD59-APT542 (250 μg in 0.1 ml of PBS), or the same volume of PBS alone (control) in a single dose given into the joint together with the disease-initiating antigen on day 0. Disease was assessed clinically.
Membrane-targeted CD59 Inhibits Antigen Arthritis

FIG. 4. *In vitro* C regulatory function of membrane-targeted CD59. The C-inhibitory activities of sCD59-Cys (open circle) and sCD59-APT542 (closed circle) were assessed in nonwash (A) and wash (B) hemolysis assays. Soluble recombinant rat CD59 expressed in CHO cells was used as positive control (sCD59; open triangle), and guinea pig erythrocytes upon which the C5b-7 complex was performed were targets. Hemolysis was assessed by release of hemoglobin to the supernatant, and inhibition of lysis was expressed as a percentage of the lysis obtained in the presence of the control protein, bovine serum albumin. Results represent the mean value ± S.D. of three determinations.

by measuring the knee diameters of inflamed (right) and noninflamed (left) knees with a Mitutoyo digital caliper over a 14-day period. On each occasion, three readings were taken from each joint by an independent observer blinded to the treatment regimen. The swelling attributed to the antigenic challenge was expressed as the difference (in mm) between the mean readings of the inflamed and noninflamed knee diameters. Results were statistically evaluated using a two-sample *t* test, and *p* values less than 0.05 were taken as significant.

Disease was also assessed histologically. Rats from different treatment groups were killed 14 days after arthritis induction. Knee joints were dissected intact and fixed in formalin-buffered saline prior to processing, sectioning, staining, and scoring for arthritis as described previously (17). The histological parameters assessed were synovial hyperplasia (on a scale of 0–3), inflammatory infiltrate (scale 0–5), intraluminal inflammatory exudate (scale 0–3), and number of cartilagenous/bony erosions (scale 0–3). The sum of the scored parameters gave the arthritis index for each knee. Results were statistically evaluated using the Mann-Whitney test for nonparametric variables, and *p* values less than 0.05 were taken as significant.

Generation of Soluble Recombinant CD59 in *E. coli*—Soluble recombinant rat CD59, comprising the first 71 amino acids of rat CD59 modified with a 7-residue carboxyl-terminal "spacer" region and terminal Cys residue, was expressed in *E. coli*. The expressed protein was present almost exclusively as inclusion bodies in the bacterial pellet, with none detected in supernatants from transfectants (data not shown). Numerous bands were present in the fermentation pellet samples, but an additional band at the predicted molecular mass for sCD59-Cys (9 kDa) was present only in the postinduction pellet sample (Fig. 1; compare lanes 1 and 2). This band was retained during several wash steps (Fig. 1, lanes 3–5), demonstrating that the protein was a component of the inclusion bodies. Upon solubilization of the bacterial pellet, it was apparent that sCD59-Cys was the major protein component (lane 6). From a 2-liter fermentational band at the predicted molecular mass for sCD59-Cys (9 kDa) was present only in the postinduction pellet sample (Fig. 2). In the prepurification sample, the polyclonal reagent detected a faint band at 9 kDa, heavier bands, presumably different conformers of dimer at 18–21 kDa, and a higher aggregate band at 48 kDa (Fig. 2A, lane 1). The ammonium sulfate pellet contained no 9-kDa monomer, but the larger bands were present (Fig. 2A, lane 2). The supernatant con-
tained predominantly the 9-kDa monomer with trace amounts of dimer remaining, demonstrating that all of the aggregated protein was contained within the pellet (Fig. 2A, lane 3). Under reducing conditions, only a 9-kDa band reactive with the polyclonal anti-CD59 antiserum was seen in each lane, indicating that all higher molecular mass bands were reduced to monomer (Fig. 2B). The mAb 6D1 (Fig. 2C) strongly stained a band at 9 kDa in the ammonium sulfate supernatant (Fig. 2C, lane 9), and, more weakly, the same band in the prepurification sample (Fig. 2C, lane 7), demonstrating that these contained correctly folded protein. No bands were stained in the ammonium sulfate pellet (Fig. 2C, lane 8), indicating that bands detected in the pellet by the polyclonal reagent represented denatured and misfolded protein. The final yield of pure, correctly refolded monomeric protein was 9 mg, as determined by Coomassie protein assay (Pierce), with a refolding efficiency of 5%. The obtained N-terminal sequence was MLRXXYNX, identical to the known sequence (MLRCYNC) with, as anticipated, failure to identify Cys residues. Retention of the initiating methionine has frequently been described in prokaryotic expression systems. The molecular mass of the refolded protein, measured using MALDI-TOF, was 9,039 Da (theoretical mass 8728 Da).

Generation and Characterization of Membrane-targeted sCD59—sCD59-Cys was modified at its C-terminus by the addition of the membrane address tag, APT542, using thiol interchange chemistry. The “tagged” protein was purified by cation-exchange chromatography; SDSPAGE analysis (Fig. 3) of soluble CD59 before (lane 1) and after (lane 2) conjugation to APT542 showed that the addition of a membrane address tag increased the apparent molecular mass of sCryn-Cys from 9 to 11 kDa. Efficiency of tailing of sCD59-APT542 was between 80 and 100%, and the residual untailed material was efficiently separated by chromatography on Mono-S (not shown). The molecular mass of the tailed protein, measured using MALDI-TOF, was 10,725 Da.

The capacity of sCD59-APT542 to inhibit MAC-mediated cell lysis was compared with that of untargeted sCD59-Cys and CHO-expressed sCD59 using nonwash (Fig. 4a) and wash (Fig. 4b) hemolysis assays on GPE5b-7 cells. In a nonwash assay, sCD59-Cys expressed in E. coli and sCD59 expressed in CHO cells (either with or without N-glycosylation), when used in equimolar amounts, were similarly effective at inhibiting lysis, indicating that the bacterially expressed and refolded protein was fully active when compared with that expressed in CHO. In contrast, sCD59 containing a membrane-targeting moiety (sCD59-APT542) was 100-fold more active than the untargeted proteins. In the wash assay, the differences were even greater. The C-inhibitory profile of sCD59-APT542 was almost identical in wash and nonwash assays, indicating that the protein had bound firmly to the cells and there exerted its effect. In contrast, no residual hemolysis inhibiting activity was seen with
Membrane-targeted CD59 Inhibits Antigen Arthritis

the unmodified soluble molecules (sCD59-Cys, sCD59) after three wash steps.

**sCD59-APT542 Therapy in Rat AIA**—In order to assess the potential of sCD59-APT542 as a therapeutic agent, its effects on the course of rat AIA were investigated. Either sCD59-Cys or sCD59-APT542 was given as a single dose of 250 μg/joint with the eliciting antigen at the time of disease induction (day 0) and compared with vehicle control. Clinical outcomes in the groups (n = 6 each) are shown in Figs. 5 and 6. sCD59-APT542-treated animals showed a dramatic reduction in clinical disease as assessed by joint swelling, which was significant when compared with vehicle-treated animals on days 2–14 (p < 0.05) (Fig. 5). Notably, the untreated sCD59-Cys-treated animals also showed significantly less joint swelling when compared with controls and were not significantly different from the sCD59-APT542 group at any time point (Fig. 5).

The effect of sCD59-APT542 on disease progression in AIA was assessed histologically in animals sacrificed on day 14 postinduction. Representative sections from untreated and treated rats are shown in Fig. 6. A–D. Untreated diseased joints displayed diffuse, dense infiltration of synovial tissue together with extensive cartilage destruction and bone erosion. Animals treated with sCD59-Cys also displayed marked inflammatory changes similar to those seen in untreated joints. In contrast, sCD59-APT542-treated knee joints retained a smooth cartilage surface with no erosions and only patchy mononuclear infiltration with minimal and focal synovial lining thickening. Joints were scored for multiple parameters of arthritis, and an overall index of severity was calculated. A single administration of sCD59-APT542 at day 0 reduced the severity score at day 14 from a mean ± S.D. of 10.7 ± 3.0 for the untreated group to means of 2.0 ± 1.26 for the sCD59-APT542-treated group and 6.0 ± 3.09 for the sCD59-Cys-treated group. Statistical analysis revealed differences between groups of sCD59-APT542 versus control, p < 0.01; sCD59-APT542 versus sCD59-Cys, p < 0.05, and sCD59-Cys versus control, p > 0.05 (not significant) by the Mann-Whitney Test (Fig. 6E). Joints were stained for MAC using a well characterized anti-rat C9 antibody (Fig. 7). MAC deposits were found in abundance in control joints along the hypertrophic synovium and in areas of infiltration. Weak and inconsistent MAC deposits were found in sCD59-Cys-treated joints, and no MAC was found in joints treated with sCD59-APT542.

**DISCUSSION**

The overall aim of this program of work is to develop novel anti-C therapies that can be utilized in both acute and chronic conditions where C activation is a driving force. The potential therapeutic value of inhibiting C activation is well illustrated by the successful use of human C regulatory proteins such as sCR1 in animal models (26, 27). However, studies to date have been limited to acute diseases such as experimental nephritis, AIA, and demyelination. This is due to numerous considerations, including the short half-lives and immunogenic nature of human C regulators in rodents, the high cost of recombinant protein expression in mammalian cells, and the potential for causing harm by chronic inhibition of C activity. In order to develop anti-C therapies that can be used in the treatment of chronic conditions, these problems must be overcome. To circumvent the problem of antigenicity, we have adopted the strategy of using rodent C regulators in rodent models. To address the short half-life, we have utilized novel membrane targeting strategies that retain the C regulator on cells either in the circulation or in tissue sites (2, 18). The issue of cost has been addressed by the development of efficient bacterial expression systems for C regulators. We have recently described the expression in bacteria of a truncated form of the rodent regulator of C activation, Crry (18). The expressed protein was fully active as a C regulator, and the addition at the carboxyl-terminus of a membrane-targeting moiety, described below, markedly increased C-inhibitory activity *in vitro* and half-life *in vivo*. However, this agent, termed sCrry-APT542, acts early in the C activation pathway, blocking formation of C opsonins and chemotactic peptides and as such will restrict the essential physiological roles of C in opsonization and bacterial killing. An agent that inhibited later in the C pathway to prevent formation of the profoundly pathogenic MAC while permitting opsonic activity would be advantageous for chronic treatment.

To this end, we have undertaken the bacterial expression of a soluble recombinant form of the rat analogue of CD59, the sole membrane regulator of MAC assembly. Soluble forms of CD59 have been expressed in a variety of eukaryotic systems and shown to have MAC-inhibitory activity *in vitro* (11, 20, 21). To date, activity *in vivo* of recombinant forms of CD59 has not been reported, and no reports of expression of CD59 in prokaryotic systems have been published. Here we describe the overexpression of a soluble form of rat CD59 in *E. coli*. Expression was optimized to obtain yields of around 50 mg/liter of bacterial culture in the fermenter. As expected with these high expression levels, the protein accumulated as dense, insoluble protein aggregates within inclusion bodies in the cells (24, 28). Isolation, solubilization, and renaturation of proteins from these aggregates in active form represents a significant hurdle that increases with increased complexity of secondary and tertiary structure in the protein. CD59 contains five disulfide bonds that lock the protein in a compact structure and are essential for C regulatory activity (29). Despite this complexity, the expressed protein (sCD59-Cys) was successfully solubilized and refolded from inclusion bodies and shown to be fully active

![Fig. 7. Staining for MAC deposition in joints.](image-url)
as an inhibitor of MAC assembly when compared with soluble rat CD59 expressed in mammalian cells. The final yield of refolded, active protein averaged only 5 mg/liter of bacterial culture over several production runs. This is lower than yields obtained in our previous reports on bacterial expression of the short consensus repeat-containing C regulatory proteins, where nearly complete refolding and final yields of about 100 mg/liter were obtained (18, 24). Attempts were made to improve the efficiency of refolding by empirical adjustments in the buffers and conditions used in this step, but no significant improvement was achieved in this study. This probably reflects the ease with which this multicysteine polypeptide can be diverted down unproductive and irreversible aggregation pathways. Further work is in progress to address this issue with both rat and human proteins. Nevertheless, sufficient active protein was produced for further studies. The protein was expressed with a seven-residue Ser-Gly “spacer” and final Cys residue at the carboxyl terminus to permit the addition of groups at this site. The Cys-tagged protein was fully active in hemolysis inhibition assays when compared with sCD59 from CHO cells. Some spontaneous dimerization of sCD59-Cys occurred upon storage, but dimer was easily separated from monomer by gel filtration.

Membrane-targeted recombinant rat CD59 was generated from sCD59-Cys by coupling a membrane address peptide (APT542), comprising a lipid moiety that interacts with the hydrophobic interior of the plasma membrane and a short positively charged peptide that interacts with negatively charged phospholipid head groups (25). Coupling to the carboxyl-terminal Cys by standard thiol interchange chemistry was highly efficient and yielded almost 100% sCD59-APT542. Residual untagged sCD59-Cys was removed by cation exchange chromatography, and the tagged and untagged agents were compared in hemolysis assays. In standard nonwash assays where agent was present throughout, the C-inhibitory activity of the sCD59-APT542 was increased over 100-fold when compared mole-for-mole with sCD59-Cys or sCD59 expressed in CHO cells. In wash assays, C-inhibitory activity of the untagged forms of sCD59 was completely lost, whereas sCD59-APT542 retained strong C-inhibitory activity even after multiple washes of the target cells, demonstrating that it had incorporated into the cell membrane. Soluble forms of CD59 have been little tested as C therapeutics primarily because of their low specific activity in the presence of serum (11, 14) and small size, probably leading to rapid elimination in urine. sCD59-APT542 overcomes these problems. As a first investigation of the capacity of this agent to inhibit C-mediated pathology in vivo, we chose to study its effect on the course of AIA in the Lewis rat. AIA is an acute monoarticular arthritis that has previously been shown to be profoundly C-dependent and susceptible to treatment with anti-C agents (17, 30). A single 250-μg dose of either sCD59-APT542 or sCD59-Cys given intra-articularly at the time of disease induction markedly inhibited disease as assessed by measurement of joint swelling, indicating that both agents influenced the acute inflammation associated with this model. However, when joint pathology was assessed histologically at end point, only sCD59-APT542 significantly influenced the disease process, indicating that retention of agent in the joint improved outcome. In previous unpublished work, we have been unable to demonstrate an effect of sCD59 expressed in CHO cells in this model and were surprised to find an effect, albeit limited to acute inflammation, in this study. It is possible that dimerization of sCD59-Cys in the joint increased half-life, although this is not proven. Nevertheless, the key finding was that sCD59-APT542 markedly inhibited the progression of disease as assessed by histology. MAC deposition was also blocked in joints treated with this agent. The degree of inhibition of disease was at least equivalent to that obtained using a membrane-targeted truncated form of sCR1 (APT-070) (17), strongly implicating the MAC as the major drive to pathology in this model and demonstrating the therapeutic potential of a powerful inhibitor of MAC function.

These encouraging studies will now be extended to situations requiring systemic administration of agent and to chronic disease models to test the prediction that sCD59-APT542 will provide prolonged and targeted delivery of MAC inhibition in C-mediated pathologies.

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