Expression of the glycosylphosphatidylinositol-linked complement-inhibiting protein CD59 antigen in insect cells using a baculovirus vector

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CD59 antigen (CD59) is a glycosylphosphatidylinositol (GPI)-linked membrane glycoprotein which protects human cells from complement-mediated lysis. Here we report the expression of functionally active CD59 in *Spodoptera frugiperda* insect cells using a baculovirus vector. Recombinant CD59 was expressed abundantly on the surface of the insect cells and protected the cells from lysis by human complement. The protein was released from the cell surface by treatment with phosphatidylinositol-specific phospholipase C, indicating that it was attached to the insect cell membrane via a GPI anchor. The cells also secreted CD59 into the culture medium. Recombinant CD59 was affinity-purified from spent culture medium and from detergent extract

of transfected cells. Protein purified from both sources produced multiple bands on SDS/PAGE, all of a lower apparent molecular mass than the human erythrocyte protein. However, N-terminal protein sequencing and deglycosylation studies confirmed that signals for leader peptide cleavage and N-linked glycosylation had been recognized in the insect cells, suggesting that the differences in apparent molecular mass between the native and recombinant proteins were attributable to the extent of glycosylation. Protein derived from both sources was, in part, GPI-anchored as demonstrated by phase-partition studies and incorporation into cells membranes. Incorporated recombinant protein rendered erythrocytes resistant to complement lysis.

INTRODUCTION

The complement system is an important part of the innate immune system, providing a first line a defence against invading micro-organisms and playing a role as a mediator of inflammation. However, inappropriate deposition of complement on the surface of host cells can lead to widespread tissue damage. This is prevented by a number of membrane-bound regulatory proteins which control complement activation at the cell surface. One such protein is CD59 antigen (CD59), a 20 kDa glycosylphosphatidylinositol (GPI)-linked glycoprotein, the simultaneous isolation of which by several groups has given rise to a number of alternative names: P-18, HRF 20, MACIF, MRL, MEM-43 (Sugita et al., 1988; Davies et al., 1989; Okada et al., 1989a; Holguin et al., 1989; Stefanova et al., 1989). CD59 inhibits the lytic activity of complement by blocking the incorporation of multiple C9 molecules into the membrane attack complex (MAC), thereby preventing the formation of functional pores in the membrane (Meri et al., 1990; Rollins and Sims, 1990). A role for CD59 in T-cell activation has also been proposed (Groux et al., 1989; Stefanova and Horejsi, 1991; Korty et al., 1991; Deckert et al., 1992; Hahn et al., 1992).

The aim of this study was to express CD59 abundantly in eukaryotic cells in order to produce sufficient protein for detailed studies of its structure and function. Although expression of CD59 has been achieved in mammalian cells (Davies et al., 1989; Philbrick et al., 1990; Walsh et al., 1991; Zhao et al., 1991; Takizawa et al., 1992), the yield of protein in these studies, where measured, was very low. We chose to express CD59 in insect cells using a baculovirus vector, as this system has been shown to achieve high levels of expression of other eukaryotic proteins (Luckow and Summers, 1988). Here we describe the expression of CD59 in this system and the characterization of the expressed protein. The expressed protein was present on the insect cell

membrane and in the culture fluid, and protein purified from both these sources was capable of inserting into cell membranes and inhibiting complement lysis, indicating that it was GPI-anchored and partially glycosylated. To our knowledge, this is the first successful expression of a GPI-linked protein in insect cells. The expression of functionally active GPI-anchored CD59 in the baculovirus system provides us with an abundant source of protein for functional and structural characterization and for assessment of therapeutic efficacy.

MATERIALS AND METHODS

Chemicals and reagents

PBS, pH 7.2, was obtained in tablet form from Oxoid (London, U.K.). Reagents for SDS/PAGE were from Bio-Rad (Richmond, CA, U.S.A.). Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was from Peninsula Laboratories (St. Helens, Merseyside, U.K.). Neuraminidase and deglycosylating enzymes were from Boehringer-Mannheim (Lewes, E. Sussex, U.K.). Prosep A was obtained from Bio-Processing (Consett, Co. Durham, U.K.). CNBr-activated Sepharose CL4B was from Pharmacia (Milton Keynes, Bucks., U.K.). Poly(vinylidene difluoride) (PVDF) Immobilon membrane was from Millipore (Watford, Herts., U.K.). All other chemicals were from Sigma Chemical Company (Poole, Dorset, U.K.) or BDH Chemicals (Poole, Dorset, U.K.).

Alternative pathway buffer (APB) contained 5 mM sodium barbitone, 140 mM NaCl, 10 mM EGTA, 7 mM MgCl₂ and 0.02 % NaN₃.

Sera, antibodies and other proteins

Normal human serum (NHS) was obtained by venepuncture from healthy volunteers and stored in portions at –70 °C. Bric

Abbreviations used: MAC, membrane attack complex; APB, alternative pathway buffer; mAb, monoclonal antibody; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI, glycosylphosphatidylinositol; PVDF, poly(vinylidene difluoride); AcNPV, *Autographica californica* nuclear polyhedrosis virus; NHS, normal human serum; FITC, fluorescein isothiocyanate; TBS, Tris-buffered saline; DAF, decay-accelerating factor.

229 [mouse anti-CD59 monoclonal antibody (mAb)] and Bric 216 [mouse anti-decay-accelerating factor (DAF) mAb] were obtained as crude culture supernatants from Bio Products Laboratory (BPL; Elstree, Herts., U.K.), and contained IgG at approximate concentrations of 86 $\mu\text{g/ml}$ and 65 $\mu\text{g/ml}$ respectively. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG was from Dakopatts (Glostrup, Denmark). Horseradish peroxidase-labelled goat anti-mouse IgG was from Bio-Rad. MEM-43 anti-CD59 mAb in the form of ascites was the kind gift of Dr. V. Horejsi (Czechoslovak Academy of Sciences, Prague, Czechoslovakia); IgG was purified from the ascitic fluid using Prosep A according to the manufacturer's instructions. Human erythrocyte CD59 was prepared as previously described (Davies et al., 1989).

Cells and virus

Spodoptera frugiperda (Sf9 and Sf21) insect cells were purchased from British Biotechnology (Cowley, Oxford, U.K.). Cells grown in monolayer were cultured at 27 °C in TNM-FH medium (Sigma) supplemented with 10 % fetal calf serum. Cells grown in suspension were cultured at 27 °C in the same medium with 0.1 % pluronic F-68 (Sigma) added to reduce shear stress caused by stirring. During protein production, the insect cells were maintained in Excell 401 protein-free medium (Sera Lab, Crawley Down, Sussex, U.K.) containing 0.1 % pluronic F-68. Cells were subcultured three times a week. *Autographica californica* nuclear polyhedrosis virus (AcNPV) was purchased from British Biotechnology. Stocks of wild-type and recombinant virus were stored as culture supernatant at 4 °C.

Construction of a CD59 cDNA-containing baculovirus transfer vector

The CD59 cDNA in the plasmid CDM8 was kindly provided by Professor H. Waldmann. This construct contained the CD59 cDNA clone YTH 53.1/3, a 540 bp insert containing the full-length CD59-coding sequence and polyadenylation signal (Davies et al., 1989). The baculovirus transfer vector pBluebac was purchased from British Biotechnology. This vector contains the AcNPV polyhedrin gene promoter and recombination sequences, as well as the β -galactosidase gene under regulation of the ETL promoter. The CD59 cDNA insert was excised from CDM8 by digestion with *Xba*I and ligated into the *Nhe*I site in pBluebac, downstream of the polyhedrin gene promoter. pBluebac/CD59 containing the cDNA insert in correct orientation was identified by PCR analysis of plasmid DNA using combinations of oligonucleotide primers corresponding to the 5' and 3' coding sequences of the CD59 gene and primers corresponding to the polyhedrin gene flanking sequences (British Biotechnology).

Generation and purification of recombinant baculovirus

Sf9 cells (2×10^6 cells in monolayer) were transfected with 2 μg of plasmid DNA and 1 μg of wild-type AcNPV DNA, by the method of Summers and Smith (1987), in order to permit homologous recombination of plasmid and viral DNA and thereby transfer the CD59 cDNA into the AcNPV genome. Culture supernatant containing putative recombinant virus was harvested 6 days after transfection, and recombinant virus was isolated by several rounds of plaque assay as described (Summer

and Smith, 1987). As recombinant virus expressed the β -galactosidase gene from the Bluebac plasmid as well as the gene of interest, X-gal (Sigma) at a final concentration of 150 $\mu\text{g/ml}$ was included in agarose overlays. Recombinant viral plaques were then identified on the basis of blue colour and the absence of polyhedra from the cells. Throughout plaque purification, putative recombinant virus was examined for the presence of CD59 cDNA in correct orientation by PCR analysis as described below.

PCR from cell extracts containing recombinant baculovirus

Recombinant viral plaques were picked and transferred into 1 ml of Sf9 cells (10^5 cells/ml) in a 24-well plate. After 2 or 3 days, cells were harvested and resuspended in 300 μl of PCR buffer (10 mM Tris, 50 mM KCl, 2 mM MgCl_2 , pH 8.3) containing 0.5 % Tween 20 and 100 $\mu\text{g/ml}$ Proteinase K. After incubation at 55 °C for 1 h, the cell lysate was heated to 95 °C for 10 min to inactivate the Proteinase K, and 10 μl of this mixture was used for PCR analysis.

Flow cytometry

Sf9 or Sf21 cells were harvested 2 or 3 days after infection and washed twice in FACS buffer (PBS/1 % BSA/0.02 % NaN_3) before resuspension at a concentration of $5 \times 10^6/\text{ml}$. Cells (100 μl) were incubated at room temperature for 30 min with 100 μl of 1:100 dilution of Bric 229 anti-CD59 mAb or Bric 216 anti-DAF mAb (control), washed twice in FACS buffer, then incubated at room temperature for 30 min with FITC-conjugated goat anti-mouse IgG at a final dilution of 1:100. The cells were then washed twice more and fixed for 15 min at room temperature in 1 % *p*-formaldehyde before analysis on a FACScan analyser (Beckton Dickinson, Abingdon, Oxford, U.K.).

PI-PLC treatment of cells

Cells were washed once in PBS and resuspended at a concentration of $5 \times 10^6/\text{ml}$. Portions (100 μl) were incubated for 20 min at 30 °C with 5 μl of PI-PLC (5 munits), after which they were washed twice in FACS buffer and stained for flow-cytometric analysis as described in the above section.

Affinity purification of recombinant CD59

Recombinant CD59 was purified from spent culture medium or whole cell extracts between 3 and 5 days after infection by affinity chromatography using MEM-43 IgG (20 mg of antibody immobilized on 15 ml of Sepharose CL-4B). Cells were washed twice in PBS before resuspension in an equal volume of PBS/1 % CHAPS and stirring for 1 h at room temperature. Insoluble material was then removed by centrifugation at 38000 *g* for 20 min, and the supernatant dialysed against PBS/0.05 % CHAPS/0.02 % NaN_3 before passage over the MEM-43 affinity column equilibrated in the same buffer. Spent culture medium was passed over the column equilibrated in PBS. In each case, the column was then washed with 1 M NaCl in PBS, and specifically bound protein was eluted with 20 mM diethylamine. Fractions (1 ml) were neutralized immediately with 1 M Tris/HCl, pH 7.0, and 10 μl samples analysed by SDS/PAGE and immunoblotting.

SDS/PAGE and immunoblotting

Samples were analysed by SDS/PAGE using 15 % gels. Protein bands were visualized by silver staining (Merill et al., 1981) or

electrophoretically transferred to nitrocellulose using miniblot apparatus (Bio-Rad) according to the manufacturer's instructions. After transfer, blots were blocked with 2.5 % non-fat milk powder in Tris-buffered saline (TBS) for 30 min at room temperature, then probed for 1 h at room temperature with Bric 229 culture supernatant diluted 1:100 in TBS, or non-immune mouse IgG at 10 µg/ml in TBS (negative control). After two 15 min washes in TBS/0.5 % non-fat milk powder, first antibody was detected using peroxidase-conjugated goat anti-mouse IgG at a final dilution of 1:1000 and chloronaphthol substrate (both from Bio-Rad).

Deglycosylation of recombinant CD59 antigen

Recombinant CD59 affinity-purified from Sf9 culture medium was treated with three glycosidases of different specificities using the protocol recommended by the manufacturer. In brief, samples of recombinant CD59 (2.5 µg in 50 µl of PBS/1 % SDS) were denatured by heating to 100 °C for 2 min, then 200 µl of 20 mM sodium phosphate buffer, pH 7.2, containing 50 mM EDTA, 0.5 % Nonidet P40 and 10 mM Na₂S₂O₃ was added and the solution heated to 100 °C for a further 2 min. The protein solution was allowed to cool to room temperature, then one or more of the different deglycosylating enzyme(s) were added as described in the Results section, in the following amounts: 1 munit (0.1 µl) of neuraminidase; 0.1 unit (8 µl) of Endo-F (a mixture of endoglycosidase F and N-glycosidase F); 1 munit (2.5 µl) of O-glycosidase. After overnight incubation at 37 °C, each sample was concentrated to 50 µl using an Ultrafree-MC filter (molecular-mass cut-off 10 kDa; Millipore), and samples were taken for analysis by SDS/PAGE and immunoblotting.

Phase separation in Triton X-114

Recombinant CD59 purified from culture medium or from insect cells was subjected to phase separation in Triton X-114 essentially as described by Hooper and Bashir (1991), either untreated or after pretreatment with PI-PLC (5 munit/ml, 30 min, 37 °C). In brief, the protein sample (100 µg/ml, 50 µl) was diluted in 200 µl of 2 % Triton X-114 in TBS at 4 °C, mixed, layered on top of 0.3 ml of 6 % (w/v) sucrose (in TBS containing 0.06 % Triton X-114) and incubated for 5 min at 37 °C. The tubes were centrifuged at 3000 g for 3 min in a benchtop microfuge. The upper aqueous phase was carefully removed, fresh Triton X-114 was added to 0.5 % (v/v), mixed and layered back on to the original sucrose cushion. After a further incubation at 37 °C for 5 min, the tubes were centrifuged as before. Aqueous and detergent-rich phases were then harvested from above and below the sucrose cushion respectively and made up to the same final volume. Recombinant CD59 in each phase was detected by dotting 2.5 µl portions on to nitrocellulose, followed by blocking and immunostaining with Bric 229 as described above for Western blotting. Blots were quantified by densitometry using a GDS2000 gel-documentation system and analysis software (UVP Ltd., Cambridge, U.K.). The percentage of the total CD59 appearing in the detergent and aqueous phases before and after PI-PLC treatment was calculated. Phase separation of CD59 purified from rat and human erythrocytes was similarly examined as positive and negative controls for the PI-PLC treatment.

Complement lysis of Sf9 cells

Sf9 cells infected with recombinant virus encoding CD59, or control cells infected with wild-type virus, were harvested 2 days after infection, washed twice in TNM-FH insect medium and

resuspended at 5×10^6 /ml in APB. Portions (5×10^6 cells in 100 µl) were incubated for 30 min at 30 °C with 100 µl of NHS serially diluted in APB, after which time cell viability was measured by exclusion of propidium iodide, assessed using flow cytometry. Percentage lysis was calculated after subtraction of background cell death in the absence of NHS. In some experiments, cells at 5×10^6 /ml were incubated for 20 min at room temperature with an equal volume of Bric 229 anti-CD59 diluted 1:5 in PBS (or Bric 216 anti-DAF as control), washed once and resuspended at 5×10^6 /ml in APB, before complement lysis assay. The incubation conditions described above were chosen to minimize spontaneous cell death.

Incorporation of recombinant CD59 antigen into guinea-pig erythrocytes

Recombinant CD59, purified from culture medium or from insect cells, or purified human erythrocyte CD59 was incubated with guinea-pig erythrocytes (10^7 cells/ml in PBS; CD59 at 5 µg/ml) for 30 min at 37 °C. After being washed, some of the cells were removed and stained for analysis of CD59 expression on the FACScan as described above. The remainder were suspended at 10^7 /ml in APB, incubated with various dilutions of human serum at 37 °C for 30 min, and haemolysis was estimated by measuring haemoglobin release into the supernatant. Controls included cells incubated with each CD59 (5 µg/ml) followed by buffer alone.

N-Terminal sequence analysis

Recombinant CD59 (20 µg) affinity-purified from Sf9 cells or culture medium was prepared for sequence analysis by SDS/PAGE under non-reducing conditions and electroblotting on to PVDF as described above. Protein bands were visualized by staining for 5 min in 0.1 % Coomassie Blue R in destain (40 % methanol/10 % acetic acid in water), followed by extensive destaining. The blot was air-dried, and the three recombinant CD59 bands were excised separately for N-terminal sequence analysis.

RESULTS

Production of a recombinant viral clone encoding CD59 antigen

AcNPV containing the CD59 gene was produced by co-transfection of Sf9 cells with wild-type AcNPV and pBluebac/CD59. Crude culture supernatant was harvested on day 6 after transfection and the presence of recombinant virus was confirmed as follows: a 10 µl sample was used to infect 10^7 Sf9 cells growing in monolayer, and 3 days after infection the expression of CD59 on the surface of the cells was measured by flow cytometry using anti-CD59 mAb (Bric 229) or anti-DAF mAb (Bric 216) as control. At this stage, all of the Sf9 cells contained visible polyhedra, whereas only 5 % of the cells stained for surface CD59, suggesting that the frequency of recombinant virus was very low (results not shown). However, after three rounds of plaque purification and assay, several clones of recombinant virus were purified to homogeneity, as judged by the absence of polyhedra in infected cells and the expression of CD59 on the surface of 100 % of the cells. One recombinant viral clone, AcNPV/CD59 no. 5, was selected for further study.

Expression of recombinant CD59 antigen by insect cells

Sf9 cells infected with purified recombinant virus (AcNPV/CD59 no. 5) stained brightly for surface CD59, while remaining negative

Figure 1 CD59 antigen is expressed on the surface of Sf9 cells and is released by PI-PLC

(a) Sf9 cells were infected with recombinant virus encoding CD59 (profiles 3 and 4) or wild-type virus as control (profiles 1 and 2), and harvested 3 days after infection. Surface expression of CD59 was then examined by flow cytometry using Bric 229 anti-CD59 mAb (profiles 2 and 4) or Bric 216 anti-DAF mAb used as a negative control (profiles 1 and 3). (b) Sf9 cells were harvested 3 days after infection with recombinant virus, then stained for CD59 using Bric 229 mAb with or without prior treatment with PI-PLC (profiles 1 and 2 respectively). Background staining of Sf9 cells by non-specific mouse IgG is shown in profile 3.

Figure 2 Recombinant CD59 antigen protects Sf9 cells against complement-mediated lysis

Sf9 cells infected with recombinant virus encoding CD59 (■, □) or control cells infected with wild-type virus (▼, ▽) were harvested 2 days after infection and tested for susceptibility to complement-mediated lysis. Before incubation in serum, cells were pretreated with Bric 229 anti-CD59 mAb (closed symbols) or Bric 216 anti-DAF as control (open symbols). Lysis of cells pretreated with buffer was equivalent to that shown for control antibody. Results are the means of triplicate determinations and bars represent S.D.s.

for DAF expression. Control cells that were infected with wild-type virus showed no fluorescence staining (Figure 1a). In contrast with the Sf9 cells, Sf21 cells infected with AcNPV/CD59 no. 5 expressed very little cell surface CD59 (results not included). However, immunoblotting of spent culture medium revealed that both cell types released a soluble form of CD59 into the culture supernatant.

Recombinant CD59 antigen is released from the surface of insect cells by treatment with PI-PLC

In human cells, CD59 is attached to the membrane by means of a GPI anchor. In order to test whether this was also the case in insect cells, Sf9 cells expressing CD59 on their surface were treated with PI-PLC. This enzyme cleaves the GPI anchor between the inositol ring and diacylglycerol embedded in the lipid bilayer, and thereby releases the attached protein from the cell surface. PI-PLC treatment of Sf9 cells caused a decrease in the amount of surface staining for CD59, shown by a shift in the fluorescence profile of the cells and a fall in the mean fluorescence from 74.4 to 24.5 units (Figure 1b). CD59 on the surface of human erythrocytes is not released by PI-PLC treatment, because of resistance of the erythrocyte GPI anchor to cleavage by PI-PLC (Ratnoff et al., 1992). As expected, the PI-PLC used in our experiments did not decrease human erythrocyte expression of CD59. This eliminates the possibility that the decrease in surface staining for CD59 on Sf9 cells was due to proteolysis by a contaminating protease in the PI-PLC preparation, and therefore demonstrates that CD59 expressed in Sf9 insect cells is attached to the membrane via a GPI anchor.

Functional activity of recombinant CD59 on the surface of Sf9 cells

In order to assess the functional activity of recombinant CD59, Sf9 cells were harvested 2 days after infection with AcNPV/CD59 no. 5 (or wild-type virus as control) and tested for susceptibility to lysis by human complement. Preliminary experiments revealed that the insect cells activated human complement in APB without the need for complement-fixing antibody, whereas they were not lysed by heat-inactivated serum or by serum diluted in EDTA-containing buffer. The cells were also temperature-sensitive, hence all incubations were carried out at 30 °C. Those cells

Figure 3 SDS/PAGE and immunoblotting of affinity-purified recombinant CD59 antigen

Samples of affinity-purified recombinant CD59 from Sf9 cells (lane 2) and culture medium (lane 3) were analysed by SDS/PAGE on 15% gels under non-reducing conditions, followed by either silver staining (a) or immunoblotting using Bric 229 (b). Lanes 1 and 4 show the positions of molecular-mass markers and human erythrocyte CD59 respectively. The same amounts of total CD59 protein were applied to each lane of each gel. The appearance of recombinant CD59 on SDS/PAGE under reducing conditions was identical with that shown.

infected with recombinant virus were far less susceptible to complement lysis than control cells (Figure 2), indicating that CD59 expressed on their surface is functionally active and protects against complement lysis. This was confirmed by the enhancement of lysis observed after preincubation of CD59-expressing Sf9 cells with Bric 229 anti-CD59 mAb, to block CD59 activity. This antibody had no effect on the lysis of control cells infected with wild-type virus (Figure 2). An irrelevant control antibody (Bric 216) had no effect on the lysis of either cell type.

Purification and characterization of recombinant CD59 antigen produced by Sf9 cells

CD59 was purified from both spent culture medium and detergent extract of Sf9 cells by affinity chromatography as described in the Materials and methods section. The cell culture was harvested between 3 and 5 days after infection, and, in all preparations, the yield of purified protein was approx. 300–500 µg of soluble CD59 (from 300 ml of culture medium) and approx. 150–200 µg of cell-extracted protein (from 2×10^8 cells). These two preparations were compared with each other and with human erythrocyte CD59 by SDS/PAGE and immunoblotting. On SDS/PAGE, recombinant CD59 ran as three discrete bands in the region of 12–18 kDa, compared with the broad 19 kDa band with a smeared 'tail' typical of the erythrocyte-derived protein (Figure 3a). The molecular mass of each of the bands was the same for CD59 purified from both Sf9 cell extract and spent culture

Table 1 Phase partitioning of recombinant and native CD59 in Triton X-114

Portions of native human erythrocyte (HuE) and rat erythrocyte (RtE) CD59 and recombinant supernatant (sr) and cell (cr) CD59, either treated or not with PI-PLC, were subjected to phase separation in Triton X-114 as detailed in the Materials and methods section. The detergent (D) and aqueous (A) phases were collected, brought to the same volume and portions (2.5 µl) dotted on to nitrocellulose. In the pre-partition experiment, portions of each CD59 (except rat) at the same protein concentration and in the same buffer were dotted. The nitrocellulose was blocked and immunostained for CD59 as detailed in Figure 3. Densitometric comparison of the dots was performed as described in the Materials and methods section. In the pre-partition study, results are expressed as a percentage of the absorbance obtained with the erythrocyte protein; in the partitioning studies the results are expressed as the percentage of total absorbance (D + A) in each phase (D or A). Results are means ($\pm$ S.D.) of triplicate blots on the same set of samples.

	srCD59	crCD59	HuECD59	RtECD59
Pre-partition	93 $\pm$ 9	85 $\pm$ 17	100 $\pm$ 14	–
– PI-PLC D	91 $\pm$ 13	72 $\pm$ 16	90 $\pm$ 8	98 $\pm$ 15
A	9 $\pm$ 4	18 $\pm$ 3	10 $\pm$ 7	2 $\pm$ 2
+ PI-PLC D	32 $\pm$ 11	20 $\pm$ 6	86 $\pm$ 11	19 $\pm$ 5
A	68 $\pm$ 15	80 $\pm$ 13	14 $\pm$ 6	71 $\pm$ 9

medium. The higher-molecular-mass bands visible on the gel (approx. 25 kDa and 50 kDa) are probably attributable to contaminating IgG heavy and light chains from the affinity column, as these bands were stained in control blots probed with anti-mouse IgG, but were not detectable in Sf9 culture supernatants. In preparations harvested on day 5, all three bands of both forms of recombinant CD59 were stained in a Western blot using Bric 229 mAb (Figure 3b). Although similar amounts of protein were loaded in each lane, and the recombinant proteins stained at least as strongly with silver as did erythrocyte CD59 (Figure 3a), the antibody staining was much weaker for the recombinant proteins than for erythrocyte CD59. Densitometry of dot-blots indicated that protein from all three sources reacted equally well with antibody (Table 1), indicating that the immunogenicity of the recombinant proteins was impaired by denaturation in SDS. In preparations harvested on day 3, the supernatant protein stained well but the cell-extracted protein stained very weakly, particularly after several days of storage at 4 °C, suggesting that it was unstable.

Cell- and supernatant-derived forms of recombinant CD59 contain GPI anchors

Recombinant CD59 isolated from cells or supernatant on day 5 was incorporated into guinea-pig erythrocytes as demonstrated by flow cytometry and was released by incubation with PI-PLC (reduction in median fluorescence of 70 % for supernatant protein and 60 % for cell-derived protein) (Figure 4a). Human erythrocyte CD59 was incorporated efficiently but was not significantly released by PI-PLC (a consequence of its modified GPI anchor), whereas rat CD59 was incorporated efficiently and was released by PI-PLC treatment (results not included). These controls demonstrate the specificity of the PI-PLC for GPI anchor cleavage and eliminate the possibility of proteolysis of incorporated CD59. Incorporation of recombinant protein from either source protected guinea-pig erythrocytes from complement lysis, although the supernatant-derived protein was more inhibitory (Figure 4b). Recombinant protein isolated from either source on day 3 was incorporated much less efficiently into guinea-pig erythrocytes and inhibited lysis much less efficiently (results not included). Pretreatment of recombinant CD59 from either source

Figure 4 Incorporation of recombinant CD59 into guinea-pig erythrocytes

(a) Analysis by flow cytometry of incorporation of recombinant CD59 from supernatant or cells into guinea-pig erythrocytes. Fluorescence histograms are shown of guinea-pig erythrocytes stained for CD59 using Bric 229 mAb after incubation for 30 min at 37 °C with 5 µg of recombinant CD59 purified from (1) Sf9 culture supernatant or (3) Sf9 cells. The effects of treatment with PI-PLC on incorporated supernatant and cell CD59 are shown in (2) and (4) respectively. In all instances background fluorescence of guinea-pig erythrocytes stained with non-immune mouse IgG after incorporation of CD59 was within the first log decade. (b) Incorporated recombinant CD59 protects guinea-pig erythrocytes against lysis by human serum. Guinea-pig erythrocytes were incubated with buffer alone (■), erythrocyte CD59 (□), cell-derived recombinant CD59 (○) or supernatant-derived recombinant CD59 (●) as detailed in the Materials and methods section. After being washed, the cells were resuspended and incubated with various dilutions of serum for 30 min before measurement of haemolysis. Results are means of triplicate determinations and bars represent S.D.s.

with PI-PLC before incubation with guinea-pig erythrocytes reduced inhibitory activity by more than 60 %, again implying that the recombinant proteins possessed GPI anchors.

Further proof of GPI-anchoring was sought by examining the temperature-induced phase transition of the recombinant proteins in Triton X-114. Before PI-PLC treatment, more than 70 % of the cell-derived protein and over 90 % of that from supernatant partitioned into the detergent-rich phase on separation, indicating hydrophobic or amphipathic properties (Table 1). After treatment with PI-PLC, protein from both sources partitioned predominantly into the aqueous phase (80 % of cell-derived, 68 % of supernatant-derived), indicating hydrophilic properties. Human erythrocyte CD59, in which the anchor is resistant to cleavage by PI-PLC, remained in the detergent phase after enzyme treatment whereas rat erythrocyte CD59, in which the

Figure 5 Recombinant CD59 antigen contains N-linked carbohydrate

Samples of soluble recombinant CD59 were incubated with buffer control (lane 2), neuraminidase (lane 3), neuraminidase plus *O*-glycosidase (lane 4), Endo-F (lane 5) or with all three enzymes (lane 6), before analysis by SDS/PAGE and immunoblotting using Bric 229 mAb. Prestained molecular-mass standards (Bio-Rad) are shown in lane 1.

anchor is susceptible to cleavage (Hughes et al., 1992), partitioned into the aqueous phase after enzyme treatment. The published method (Hooper and Bashir, 1991) examined phase separation of proteins from cell membranes and reported that GPI-anchored molecules were found in a detergent-insoluble pellet. In our studies, in which purified native and recombinant proteins were used, no detergent-insoluble pellet was formed. However, the shift from detergent to aqueous phases on PI-PLC treatment strongly suggests the presence of a GPI anchor in the recombinant proteins.

N-Terminal protein sequence of recombinant CD59

To confirm the identity of the three bands present in preparations of recombinant CD59 antigen, and to check that correct N-terminal processing had been carried out, protein sequence analysis was performed on CD59 purified from Sf9 cells and culture medium. All three bands in proteins from both sources had N-terminal protein sequences (to residue 12 or beyond) identical with that of the native protein. In the case of the supernatant protein, sequence data were obtained beyond Asn-18 for the upper two bands, which in native CD59 is a site of N-linked glycosylation (Okada et al., 1989a; Stefanova et al., 1989; Davies et al., 1989; Ninomiya et al., 1992). No signal was obtained for this position in the recombinant protein, confirming that here the glycosylation site is also occupied.

Deglycosylation of recombinant CD59

The apparent difference in molecular mass between native and recombinant CD59 might be partly attributable to a difference in the nature and extent of glycosylation. In order to assess the nature of the carbohydrate attached to the recombinant protein, CD59 purified from Sf9 cell culture medium was treated with neuraminidase alone or in combination with *O*-glycosidase, to remove *O*-linked carbohydrate, with a mixture of endo-glycosidase F and *N*-glycosidase F, to remove N-linked carbohydrate, or with a combination of all the above enzymes. Digested samples were analysed by SDS/PAGE and immunoblotting (Figure 5). Treatment with neuraminidase and/or *O*-glycosidase had no effect on the mobility of recombinant CD59 on SDS/PAGE. In contrast, treatment with endoglycosidase F/*N*-

glycosidase F caused a decrease in the apparent molecular mass to approx. 12 kDa, with a second less-distinct band apparent at approx. 10 kDa. The deglycosylated protein was still detectable with Bric 229 mAb anti-CD59, indicating that the epitope recognized by this antibody was not dependent on the presence of carbohydrate.

DISCUSSION

Despite its relatively recent discovery, the role of CD59 as a potent inhibitor of the complement MAC is now well established. However, there is still only limited information available regarding the molecular interactions through which CD59 exerts its biological effects. The aim of this study was to express high levels of functional CD59 in eukaryotic cells in order to provide an abundant source of material for further study. Here we describe the expression of CD59 in Sf9 insect cells using a baculovirus vector and the characterization of the recombinant protein.

Sf9 cells infected with recombinant virus expressed large amounts of cell-surface CD59 and also secreted CD59 into the medium. The infected cells stained brightly for CD59 using monoclonal antibodies raised against the native protein. Expressed CD59 was attached to the insect cell surface by means of a glycolipid anchor, as demonstrated by its release on treatment with PI-PLC. Recombinant CD59 was purified from both cell extracts and supernatants. Protein from both sources was, at least in part, GPI-anchored as demonstrated by its incorporation into membranes and its partitioning behaviour in Triton X-114 before and after treatment with PI-PLC. The recombinant protein from both sources appeared as multiple bands on SDS/PAGE, all of higher mobility than the erythrocyte protein, apparently a consequence of differences in glycosylation. Protein sequencing demonstrated that all bands in the secreted protein and in the cell-derived protein had the correct N-terminal sequence, and the apparent absence of a residue at Asn-18 in the secreted protein indicated that it was *N*-glycosylated. The expressed CD59 was functionally active, inhibiting complement lysis both on the insect cells and after purification from either cells or culture supernatant and incorporation into guinea-pig erythrocytes.

To our knowledge, this is the first demonstration of the expression of a GPI-linked protein in insect cells using the baculovirus system. It has recently been reported that insect cells infected with a recombinant baculovirus encoding the GPI-linked molecule CD58 (LFA-3) expressed CD58 on their surface, and secreted a soluble form of CD58 which contained an intact C-terminal domain in place of the GPI anchor (Albert-Wolf et al., 1991). It was suggested that the Sf9 cells may have been inefficient at processing recombinant CD58 or were unable to synthesize GPI anchors. However, in that study the susceptibility of the insect cell surface CD58 to release by PI-PLC was not examined, so it is not possible to ascertain whether the membrane-bound form of CD58 was GPI-linked. Here we show that surface-expressed recombinant CD59 is released by PI-PLC, indicating that it is anchored via GPI. Further, we show that at least a proportion of the secreted form of recombinant CD59 possesses a GPI anchor and is capable of being incorporated into membranes and protecting against complement lysis. Recombinant CD59 purified from cell extracts was also incorporated into membranes and protected cells, albeit less efficiently than the supernatant-derived protein. This relative inefficiency might be due to a proportion of this protein being intracellular in origin and not correctly anchored or fully folded. Incorrect or incomplete folding of cell-derived recombinant CD59 might also

account for the instability of this protein on storage. Freshly purified cell- and supernatant-derived protein reacted equally well with anti-CD59 on Western blots, albeit less strongly than an equivalent amount of erythrocyte protein. However, cell-derived protein stored at 4 °C rapidly lost its ability to react with anti-CD59 in Western blots and its functional activity, whereas supernatant-derived protein was stable in both respects.

A second potential difference between native CD59 and the recombinant protein is in the extent of glycosylation. The deduced amino acid sequence for CD59 contains a single potential N-linked glycosylation site at Asn-18, and failure to detect a signal for Asn-18 during sequencing confirms that this site is occupied in the mature protein (Sugita et al., 1988; Okada et al., 1989b; Davies et al., 1989). Enzymic deglycosylation of purified erythrocyte CD59 reduces its molecular mass from 18–20 kDa to 14 kDa and eliminates inhibitory activity (Stefanova et al., 1989; Okada et al., 1989a; Ninomiya et al., 1992; Van den Berg et al., 1993). The failure to detect a signal for Asn-18 in both higher-molecular-mass species of the secreted recombinant protein suggests that they also are glycosylated at this site. Insect cells are known to perform N-linked glycosylation at authentic sites, but, as endogenous proteins and some recombinant proteins have been found to contain only high-mannose-type oligosaccharides, it has been generally accepted that they lack glycosyl- and sialyl-transferases responsible for converting N-linked high-mannose oligosaccharide precursors into more complex carbohydrate structures which are probably essential for CD59 activity (Emery, 1991; Ninomiya et al., 1992). However, it has been demonstrated more recently that AcNPV-infected insect cells can in fact assemble complex N-linked carbohydrate, but that this ability is dependent on AcNPV infection (Davidson et al., 1990, 1991; Davidson and Castellino, 1991a,b). Efficiency of processing of complex carbohydrate increases with time after infection, thus it can be expected that multiple forms will be produced bearing different amounts of carbohydrate. This may partly account for the multiple banding pattern that we have consistently found on SDS/PAGE with recombinant CD59 and may also explain the finding that protein harvested from cells 5 days after infection exhibits much more activity than protein harvested from cells 3 days after infection. Enzymic deglycosylation resolved recombinant CD59 into a single major band of approx. 12 kDa, with a second less distinct band at approx. 10 kDa. This latter molecular mass is close to that predicted for the protein core of CD59 from the deduced sequence based on the cDNA, and may represent deglycosylated CD59 which also lacks a GPI anchor.

We have demonstrated that it is possible to produce active recombinant GPI-anchored CD59 in reasonable quantities in the baculovirus system. Glycosylation, although not identical with that of the native protein, is sufficient for activity. We are currently optimizing the expression system to improve our yields of protein further in order to obtain sufficient material for detailed structural and functional analysis and to begin to explore the therapeutic potential of CD59.

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