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The sheep analogue of human CD59: purification and characterization of its complement inhibitory activity

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SUMMARY

An inhibitor of the membrane attack complex of complement was isolated from the membranes of sheep erythrocytes. Fast protein liquid chromatography (FPLC) and affinity purification procedures for this sheep complement-inhibiting protein (SCIP) both yielded a pure protein with an apparent M_r of 19,000 under reducing and non-reducing conditions. Incubation of the denatured protein with neuraminidase and Endo-F reduced the apparent M_r to 18,000 and 15,000 respectively, while treatment with *O*-deglycosidase or phosphatidylinositol-specific phospholipase C (PIPLC) did not affect the apparent M_r. SCIP was detectable on erythrocytes and lymphocytes but not on platelets and could partially be removed by PIPLC treatment. Deglycosylation of the pure protein markedly reduced and PIPLC treatment abolished its activity. A monoclonal antibody (mAb) raised against sheep complement-inhibiting protein (SCIP) enhanced the susceptibility of sheep erythrocytes to lysis by homologous complement. SCIP inhibited complement after the stage of C5b–7 formation. Amino-terminal protein sequence was obtained and was shown to be similar to that of human CD59. All these features suggest that SCIP is the sheep equivalent of human CD59. Human CD59 has been reported to be species selective in that it inhibits complement from relatively few species. However, SCIP efficiently inhibited lysis of guinea-pig erythrocytes by complement from a wide range of species tested indicating that it is a potent and non-selective inhibitor of the membrane attack complex of complement (MAC).

INTRODUCTION

During the last few years the family of C-inhibiting proteins on human cells has been expanded by the discovery of two proteins which inhibit membrane attack complex (MAC) assembly and function (reviewed in ref. 1). On most human cells, the primary MAC-inhibiting factor is CD59, an 18,000–20,000 MW protein attached to the membrane by a glycosyl phosphatidylinositol (GPI) anchor.[2–5] CD59 inhibits MAC assembly by incorporation in the complex at the stage of C5b–8 thereby restricting recruitment and insertion of C9.[6,7] CD59 has also been reported to play a role in T-cell activation.[8,9] Consistent with this is the observed sequence homology of CD59 with murine Ly-6 antigens, a family of leucocyte surface molecules involved in T-cell activation.[5]

Abbreviations: APB, alternative pathway buffer; C, complement; CVF, cobra venom factor; E, erythrocyte; MAC, membrane attack complex of complement; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PIPLC, phosphatidylinositol-specific phospholipase C; SCIP, sheep complement-inhibiting protein; VSB, veronal saline buffer.

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We have recently isolated the rat equivalent of CD59 and shown that this protein closely resembled human CD59 in its inhibitory activity, linkage to the membrane, M_r and deglycosylation pattern and amino terminal amino acid sequence.[10] We were impelled to investigate the existence of sheep equivalents of CD59 because incorporation of human CD59[6] or rat CD59 (C. W. van den Berg, R. A. Harrison and B. P. Morgan, unpublished experiments) into sheep E causes much less effective inhibition of lysis by serum than does incorporation of these proteins into other target cells. This observation suggested to us that sheep E might possess a CD59-like molecule which is species non-selective and mediates protection against MAC from a wide range of species. We therefore set out to identify and characterize the sheep equivalent of human CD59.

A sheep complement inhibitory protein (SCIP) was isolated initially by a multi-column fast protein liquid chromatography (FPLC) procedure and, after production of monoclonal antibody (mAb), by immunoaffinity chromatography. SCIP closely resembled human CD59 in terms of structure and function. In accord with our initial hypothesis SCIP efficiently inhibited target cell lysis by human and rat sera. SCIP also inhibited lysis by all other sera tested. In this respect SCIP differed from human CD59 which is species selective in that it inhibits human and primate sera efficiently and sera from other species poorly or not at all.[3,5,11–13]

MATERIALS AND METHODS

Blood and sera

Sheep and guinea-pig erythrocytes (ShE and GpE) and sera and rat and mouse sera were obtained from the animal facilities of the University of Wales College of Medicine (Cardiff, U.K.). All other sera were obtained from Serotec (Kidlington, Oxford, U.K.). Sera were kept at −70°. Erythrocytes, stored 1/2 in Alsever's old solution (114 mM citrate, 27 mM glucose, 72 mM NaCl, pH 6·1) at 4°, were washed three times with PBS before use.

Buffers

Veronal saline buffer (VSB): 2·8 mm barbituric acid, 145·5 mM NaCl, 0·8 mM $MgCl_2$, 0·3 mm $CaCl_2$, 0·9 mM Na barbital, pH 7·2 (Oxoid Ltd, Basingstoke, U.K.); alternative pathway buffer (APB): 5 mM Nabarbital, 7 mM $MgCl_2$, 10 mM EGTA, 150 mM NaCl, pH 7·4; phosphate-buffered saline (PBS): 8·1 mM Na_2HPO_4, 1·5 mM KH_2PO_4, 137 mM NaCl, 2·7 mM KCl, pH 7·4 (Oxoid Ltd); EDTA–PBS: PBS + 10 mM EDTA, pH 7·4; lysis buffer: 5 mM Na phosphate, 2 mM EDTA, 1 mM benzamidine, 1 mM PMSF, 0·05% Na azide, pH 7·4.

Chemicals and enzymes

Chemicals and enzymes were purchased from the following firms: 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS), Lubrol-PX, Nonidet P-40 (NP-40), unstained SDS–PAGE M_r markers, Histopaque-1077 Sigma Immunotype mouse monoclonal isotyping kit and Cobra Venom [Sigma V9125, *N.naja kaouthia* from which cobra venom factor (CVF) was purified according to the method of Vogel and Müller-Eberhard][14] from Sigma (St Louis, MO); phosphatidylinositol-specific phospholipase C (PIPLC) (from *Bacillus thuringiensis*) from Peninsula Lab. Europe Ltd (St Helens, U.K.). Deglycosylating enzymes: Endo-F (from *Flavobacterium meningosepticum*), neuraminidase (from *Arthobacter ureafaciens*) and *O*-glycosidase from Boehringer Mannheim GmbH (Mannheim, Germany); fluorescein isothiocyanate (FITC) and horseradish peroxidase (HRP)-labelled GAM/IgG, 4-chloro-1-naphtol, acrylamide, bisacrylamide and prestained SDS–PAGE M_r markers from BioRad (Hemel Hempstead, U.K.). Coomassie and micro BCA protein assay reagents were from Pierce (Rockford, IL); amboceptor 6000 from Boehringwerke AG (Marburg, Germany); immobilized protein A (Prosep-A beads) from Bioprocessing Limited (Durham, U.K.). All other columns and column materials were obtained from Pharmacia LKB (Uppsala, Sweden).

Isolation of the sheep inhibitor

Sheep ghost preparation. Sheep erythrocyte ghosts were prepared using the method previously described for human erythrocytes.[5] Briefly, sheep erythrocytes were washed three times with PBS and resuspended in lysis buffer (1/40 v/v). After 20 hr stirring at 4° the lysed cells were concentrated and washed with lysis buffer in a Pellicon ultrafiltration system (Millipore, Harrow, U.K.) using a filter with a 300,000 MW cut-off, followed by centrifugation at 12,000 *g* for 30 min. The final volume of ghosts was 75% of the original volume of packed erythrocytes. The ghosts were 1/5 diluted in PBS and *n*-butanol (final concentration 20% v/v) was added. After stirring (30 min, 20°) and centrifugation (10 min, 20°, 1400 *g*) the aqueous phase was dialysed to the starting buffer of the first column to be used.

FPLC purification procedure. Butanol extract (50 ml, equivalent to 13 ml of packed cells) was dialysed to 20 mM Tris–HCl/0·05% CHAPS, pH 8·4 and applied to a Q-Sepharose fast-flow column (1·6 × 15 cm) equilibrated in the same buffer. Protein was eluted with 200 ml of a linear NaCl gradient (0–1 M). Fractions were tested in the CVF-reactive lysis assay as described later. Fractions containing inhibitory activity were pooled, dialysed to 40 mM Na phosphate/0·05% CHAPS, pH 7·4 and applied to a MonoQ (HR5/5) anion-exchange column, equilibrated in the same buffer. Protein was eluted with 20 ml of a linear NaCl gradient (0–0·5 M). Active fractions were pooled and $(NH_4)_2SO_4$ added to a final concentration of 1·7 M. This pool was applied to a Phenylsuperose (HR5/5) column, equilibrated in 40 mM Na phosphate, 1·7 M $(NH_4)_2SO_4$, pH 7·4. The column was washed with 40 mM Na phosphate, pH 7·4 and the inhibitor was eluted with a 20-ml linear gradient of CHAPS (0–1%) in 40 mM Na phosphate, pH 7·4.

Affinity purification procedure. An immunoaffinity column was made by coupling monoclonal antibody S75 (1 mg/ml gel, see later; column volume 4 ml) to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. CHAPS, Lubrol-PX or NP-40 were added as detergents to all separation buffers in a final concentration of 0·05%. The butanol extract (20 ml, equivalent to 5 ml of packed cells) was dialysed against PBS/detergent and applied to the column. After washing with PBS/detergent/1 M NaCl, the inhibitor was eluted with 20 mM diethylamine, pH 11·5, containing detergent and immediately neutralized with 1 M Tris–HCl, pH 7·0.

Monoclonal antibody production

Monoclonal antibodies to SCIP were produced by standard methods.[15] Briefly a BALB/c mouse was immunized i.p. with 0·5 ml of a 1:1 mixture of a 20% sheep ghost suspension and complete Freund's adjuvant (CFA), and twice boosted i.p. with 1 μg FPLC-purified SCIP with a 1-month interval between immunizations. Three days after the last i.p. booster injection the spleen cells were harvested and fused with P3/X63/AG8-653 myeloma cells (European Cell Culture Collection, Porton Down, U.K.). Positive wells were selected for in an ELISA using FPLC-purified SCIP (0·1 μg/ml) coated on soft polyvinyl chloride microtitre plates (Titertek activated, Flow Laboratories, Zwanenburg, The Netherlands) and subcloned by limiting dilution. One clone, positive after the second screening, was chosen for further study. The isotype of the clone S75 thus obtained, was IgG1. The IgG was isolated from tissue culture supernatant of clone S75 using a Prosep-A column.

SDS–PAGE

Purity of the protein was assessed on a 15% polyacrylamide gel,[16] stained with silver.[17] Western blotting was carried out on nitrocellulose using 4-chloro-1-naphthol as a developing reagent.

Protein assays

The Coomassie protein assay was used to determine the concentration of the mAb and the micro-BCA protein assay was used to estimate inhibitor concentrations in detergent-containing buffers. Bovine serum albumin (BSA) was used as a standard.

Functional assays

Inhibition assay. Inhibition of lysis of GpE by SCIP was measured in a CVF-reactive lysis assay (1) and in a rat C5b–7 site assay (2):

(1) CVF-reactive lysis assay: 20 μl of an inhibitor-containing solution was mixed with 50 μl of a 2% GpE suspension in PBS/ 0·05% CHAPS and incubated for 30 min at 37°. After centrifugation the cells were resuspended in 50 μl APB and 100 μl of a serum dilution + CVF (final concentration 2 μg/ml) in APB were added. After incubation for 30 min at 37°, unlysed cells were spun down and the absorbance of the supernate was read at 414 nm.

(2) Rat-C5b-7 site assay: a 1% GpE suspension in APB was mixed with 1/20 diluted C8-depleted rat serum (prepared by passage of rat serum over a polyclonal anti-rat C8-column) and CVF (final concentration 4 μg/ml) and incubated for 1 hr at 37°. The EC5b–7 cells were washed twice and resuspended to 1% in PBS/0·05% CHAPS. Fifty microlitres of this suspension was mixed with 20 μl of an inhibitor-containing solution and incubated for 30 min at 37°. After centrifugation the cells were resuspended in 50 μl EDTA–PBS and mixed with 100 μl of a serum dilution in EDTA–PBS. After incubation for 30 min at 37°, unlysed cells were spun down and the absorbance of the supernate was read at 414 nm. In each case, inhibition was expressed as the reduction of haemolytic activity compared to control cells incubated with buffer alone.

Effects of a monoclonal antibody against the sheep inhibitor. The effect of monoclonal antibody S75 on lysis of ShE by sheep serum was examined in two different lytic systems. In the classical pathway system ShE (2% in VSB) were mixed with amboceptor (1/400 diluted) and incubated for 10 min at 20°. The cells were washed twice, resuspended and mixed with various concentrations of S75. After 30 min incubation at 20°, the cells were washed twice, resuspended in VSB and 50 μl of the cells were mixed with 100 μl of sheep serum diluted 1/4 in VSB. After incubation for 30 min at 37° lysis was read at 414 nm. The effects of S75 on lysis of ShE-bearing preformed human C5b–7 sites was also examined. ShEC5b–7 were made by sensitizing ShE with an IgM monoclonal anti-sheep erythrocyte antibody (MAS 12b; Seralab Ltd, Crawley Down, U.K.) and then incubating the washed cells with C8-depleted human serum (diluted 1/10 in VSB, 37° for 60 min). The ShEC5b–7 cells so formed were washed and incubated with various concentrations of S75. The cells were again washed and incubated with sheep serum (diluted 1:200 in EDTA–PBS) as a source of C8 and C9, for 30 min at 37°. Lysis was measured as described above.

Deglycosylation and PIPLC treatment

Deglycosylation of SCIP (20 μg/ml, 100 μl), denatured by boiling in 1% SDS and 0·1% NP-40, was carried out with Endo-F (100 mU), neuraminidase (2 mU) and *O*-deglycosidase (0·5 mU) for 40 hr at 37° according to the manufacturer's instructions. Denatured SCIP was also treated with 20 mU PIPLC at 37° for 40 hr. For functional studies of the deglycosylated protein boiling in SDS and NP-40 was omitted.

Radiolabelling of SCIP and measurement of binding to GpE

SCIP (100 μg/ml, 0·5 ml) was labelled with ^{125}I using derivatized chloramine T (Iodobeads, Pierce and Warriner, Chester, U.K.) according to the manufacturer's instructions. Free iodine was removed by gel filtration. The specific activity of the labelled protein was 0·5–1·0 μCi/μg. Portions (0·05 ml, 20 μg/ml) were subjected to deglycosylation and PIPLC treatment essentially as described above except that the denaturation steps were omitted and the PIPLC treatment was for 4 hr only. SCIP was also incubated in the absence of enzymes for 4 and 40 hr as controls for the PIPLC treatment and deglycosylation respectively. The efficiency of deglycosylation was assessed by SDS–PAGE of aliquots of the preparations followed by autoradiography and densitometric scanning.

Triplicate aliquots (10 μl, containing 0·2 μg SCIP, approx. 300,000 c.p.m. radioactivity) of each of the above were then incubated with GpE (0·2 ml, 2·5%) for 30 min at 37°. The cells were washed three times in PBS and bound radioactivity measured. Non-specific binding of iodinated protein to GpE was assessed by addition of [^{125}I]BSA (300,000 c.p.m.) to GpE, incubating and washing as above.

The residual activity in the incorporated SCIP was assessed by resuspending the labelled cells in APS and incubating portions with CVF (3 μg/ml) and dilutions of sheep serum between 1/10 and 1/160.

FACScan analysis

Sheep blood was fractionated on Ficoll by centrifugation at 750 ***g*** for 1 hr at 20°. Lymphocytes and erythrocytes thus separated were washed in PBS. Platelets were separated from other cells by centrifugation of blood at 600 ***g*** for 10 min at 20° to obtain platelet-rich plasma, followed by centrifugation at 1400 ***g*** for 10 min at 20° to pellet platelets. Cells (100 μl at 10^7/ml) were incubated with 100 μl mAb S75 (10 μg/ml) or control mouse IgG (10 μg/ml). Fc receptors on lymphocytes were blocked with 10 μg/ml heat-aggregated human IgG. GAM/IgG.FITC was used as a second antibody. PIPLC treatment was carried out with 10 mU PIPLC for 30 min at 37°.

Amino terminal sequence analysis

Affinity-purified SCIP was prepared for sequence analysis by dialysis against 0·01 M sodium borate, 0·05% SDS, pH 8·0. Aliquots containing about 20 pmol protein (in 25–70 μl) were then applied to Biobrene^{tm}-coated filters (Applied Biosystems) and amino terminal sequence analysis was performed using an Applied Biosystems 477A protein sequencer equipped with a 120A analyser and 610A data analysis software.

RESULTS

FPLC purification procedure

The SCIP was isolated from the membranes of ShE using a multi-step FPLC procedure. A butanol extract of sheep ghosts was fractionated sequentially on Q-Sepharose fast-flow anion exchange, MonoQ anion exchange and two passages over a Phenylsuperose hydrophobic interaction column. Fractions were tested for the capacity to inhibit lysis of GpE by CVF-activated sheep serum. The Phenylsuperose column yielded, after the second passage, a pure protein with an apparent M_r of 19,000 (Fig. 1a, Table 1). The yield was 7 μg/100 ml butanol extract and the purification factor was 1667-fold from butanol extract to pure protein. The yield of more than 100% inhibitor is explained by the presence of lysis-enhancing components in the butanol extract.[10]

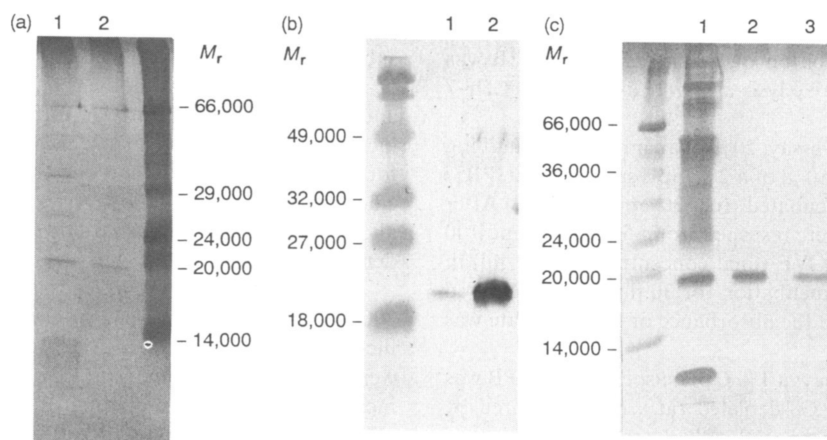

Figure 1. (a) Silver-stained gel (15% SDS–PAGE) under non-reducing conditions of Phenylsuperose-purified SCIP after the first (1) and second (2) passage. (b) Western blot of butanol extract of sheep ghosts (1) and Phenylsuperose fraction (2) separated on 15% SDS–PAGE under non-reducing conditions. The blot was probed with 10 mg/ml mAb S75. (c) Silver-stained 15% SDS–PAGE under non-reducing conditions of affinity-purified SCIP using CHAPS (1), NP-40 (2) and Lubrol-PX (3) as detergents.

Table 1. Specific activity, yield and purification rate of SCIP at various stages of the FPLC purification and affinity-purification procedure

	Specific activity (U/mg)	Yield (%)	Purification rate
Butanol extract	0·069	100	1
Q-Sepharose	0·084	81	1·2
MonoQ	0·32	134	4·6
Phenylsuperose 1st run	15	38	217
Phenylsuperose 2nd run	115	115	1667
Affinity column	142	443	2366

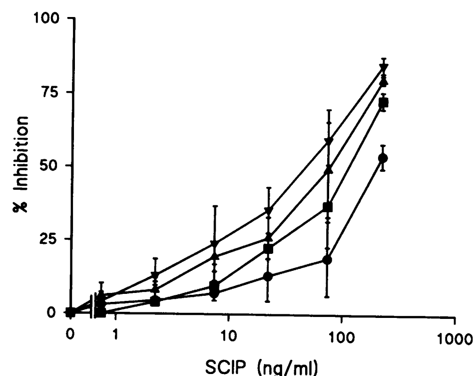

Figure 2. Dependence of inhibition on dose of sheep serum. SCIP was incubated with a 2% suspension of GpE, the cells were washed and exposed to CVF and different dilutions of sheep serum [1/15 (●); 1/20 (■); 1/30 (▲); 1/40 (▼)]. Each point represents the mean of duplicate determinations (±SD).

Affinity-purification procedure

The mAb S75 raised and selected against FPLC-purified SCIP recognized a single band with an apparent M_r of 19,000 on a Western blot of the butanol extract and the Phenylsuperose fraction (Fig. 1b). None of five available mAb recognizing human CD59 and none of the nine mAb recognizing rat CD59 cross-reacted with SCIP; polyclonal antisera against these proteins also did not cross-react with SCIP (data not shown). S75 coupled to CNBr-activated Sepharose was used as an affinity matrix to purify the sheep inhibitor more efficiently. Passage of a butanol extract over this column followed by washing and elution in buffer containing CHAPS yielded an impure preparation containing many proteins in addition to SCIP whereas when Lubrol-PX or NP-40 were used as detergent, pure protein was eluted (Fig. 1c). In subsequent purifications Lubrol-PX was used in all buffers. The yield of the purification was 240 μg/100 ml butanol extract with a purification factor of 2366-fold (Table 1).

Inhibitory activity of affinity-purified SCIP

The affinity-purified protein was incubated with 2% GpE, unincorporated protein was washed off and cells were exposed to sheep serum using a CVF-reactive lysis assay. The inhibition was dependent on the amount of sheep serum and SCIP offered (Fig. 2). One unit of SCIP was defined as the amount of protein giving rise to 50% inhibition of lysis by a dose of serum which lysed 50% of a 2% suspension of GpE in the absence of inhibitor. According to this definition the specific activity of affinity-purified SCIP was 142 U/μg (Table 1).

Deglycosylation/PIPLC treatment

The affinity-purified SCIP was treated with *N*- and *O*-reactive deglycosylating enzymes and PIPLC (Fig. 3). Treatment with Endo-F, which removes N-linked carbohydrate, resulted in a reduction in apparent M_r of 4000 (lane 2), neuraminidase reduced the M_r by approximately 1000 (lane 3), while *O*-deglycosidase did not affect the M_r (lane 4). Treatment with Endo-F together with neuraminidase did not reduce the M_r further but the efficiency of deglycosylation by Endo-F was enhanced (lane 5). Monoclonal antibody S75 recognized the deglycosylated protein on Western blot (lanes 2 and 5). PIPLC

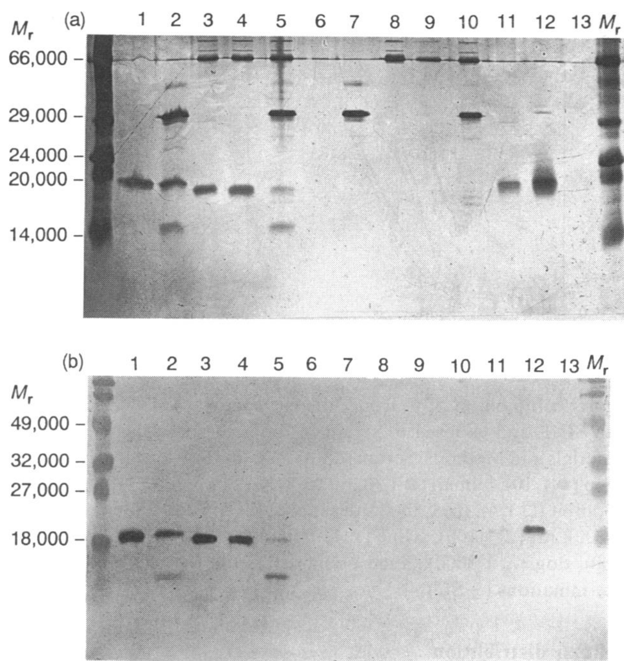

Figure 3. Silver-staining (a) and Western blot (b; probed with S75; 10 mg/ml) of 15% SDS-PAGE under non-reducing conditions of SCIP treated with deglycosylating enzymes or PIPLC: lanes 1-5, 11, 12: SCIP; lanes 2, 7, Endo-F; lanes 3, 8, neuraminidase; lanes 4, 9, neuraminidase and *O*-deglycosidase; lanes 5, 10, neuraminidase, *O*-deglycosidase and Endo-F; lanes 11, 13, PIPLC. Identical amounts of SCIP were loaded in all lanes except those containing enzymes alone.

treatment of SCIP did not affect the M_r (lane 11) but the PIPLC-treated protein stained less well with silver and blotted less strongly with the antibody S75. These findings may be artefacts due to inefficient fixation of the PIPLC-treated protein, a possibility supported by the observation that dot blots of PIPLC-treated and untreated SCIP stained equally strongly with S75 (data not included). All the above enzymes were stated by the manufacturers to be free of contaminating proteases and no degradation products suggestive of proteolysis were observed on SDS-PAGE. Efficient deglycosylation was also achieved without denaturation enabling the effects of these treatments on inhibitory activity to be assessed. Removal of N-linked carbohydrate using Endo-F reduced activity but did not completely inactivate the protein, presumably a consequence of incomplete deglycosylation, whereas removal of the GPI anchor using PIPLC caused complete loss of activity (Fig. 4). Neuraminidase treatment had no effect on activity and the protein was also heat stable as shown by its retention of activity after incubation for 5 min at 100° (Fig. 4).

In order to investigate the effects of these treatments on incorporation of SCIP into target cells ^{125}I-labelled SCIP was subjected to deglycosylation and PIPLC treatment as described above prior to incubation with GpE. The results are summarized in Table 2. Deglycosylation did not reduce the binding of [^{125}I]SCIP to GpE. In contrast, PIPLC treatment reduced binding by over 60%. The inhibitory activity was, in both cases, markedly reduced. Deglycosylation, as assessed by SDS-PAGE and autoradiography, was incomplete, about 60% of the protein

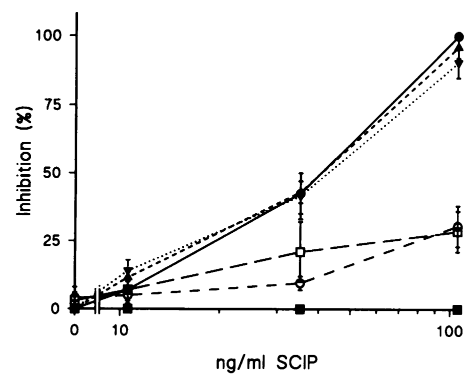

Figure 4. Inhibition of lysis of GpE by SCIP after incubation of SCIP for 5 min at 100° (▲), or treatment with PIPLC (20 mU) (□), neuraminidase (2 mU) (▼), Endo-F (100 mU) (□) or neuraminidase plus Endo-F (○). All incubations were at 37° for 40 hr. Untreated SCIP, incubated under identical conditions, served as a control (●). Each point represents the mean of duplicate determinations (±SD).

Table 2. Binding and activity of radiolabelled SCIP on GpE after deglycosylation and PIPLC treatment*

Treatment	Binding (%)†	Inhibition (%)‡
Control 1§	24·3	38·25
Endo-F	25·3	15·62
Neuraminidase	24·0	34·45
Endo-F + Neuraminidase	24·1	5·87
Control 2¶	28·5	39·24
PIPLC	11·5	12·37

* SCIP was radiolabelled and subsequently deglycosylated or PIPLC treated as described in Materials and Methods.

† Percentage of added SCIP binding to GpE was calculated after subtraction of non-specific binding of radiolabel; results are the means of quadruplicate determinations for each treatment.

‡ Percentage inhibition of lysis consequent upon incorporation of SCIP was measured in duplicate for three serum dilutions (1/10, 1/20, 1/40); results are the means of inhibition compared to appropriate controls at each serum dilution.

§ Control 1, SCIP incubated at 37° for 40 hr in the absence of enzymes (deglycosylation control).

¶ Control 2, SCIP incubated at 37° for 4 hr in the absence of enzymes (PIPLC treatment control).

running as a 14000 MW band upon treatment with Endo-F alone and 80% after treatment with Endo-F plus neuraminidase. It is thus likely that the deglycosylated protein was completely inactive, residual activity correlating closely with residual undigested SCIP. PIPLC treatment in this set of experiments was also incomplete as evidenced by residual incorporation of SCIP and inhibitory activity (each about 30–40%). In other experiments PIPLC treatment caused complete loss of activity (Fig. 4).

Enhancement of lysis of sheep erythrocytes by mAb

The effect of mAb S75 on the lysis of the ShE by homologous complement was studied. Classical pathway conditions had to

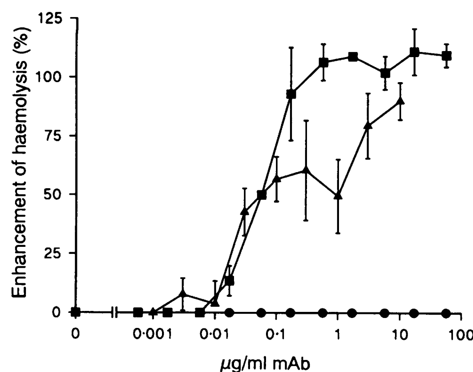

Figure 5. Enhancement of lysis of sheep erythrocytes after incubation with mAb S75. Sensitized ShE (■) or ShEC5b-7 cells (▲) were incubated with different concentrations of mAb S75 and washed. ShE were then exposed to sheep serum diluted 1/4 in VSB and ShEC5b-7 to sheep serum diluted 1/200 in EDTA-PBS. Controls were unsensitized ShE incubated with mAb S75 followed by sheep serum diluted in VSB (●). Each point represents the mean of duplicate determinations (+SD).

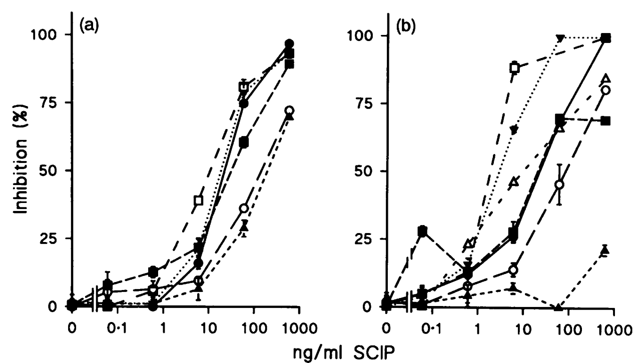

Figure 7. Inhibition of C from different species. GpE were incubated with SCIP and exposed to serum of different species as described in Materials and Methods. Serum (dilutions): (a) CVF-reactive lysis assay: sheep (● 1/10); human (■ 1/6); rat (▲ 1/100); swine (▼ 1/40), goat (○ 1/6); rabbit (□ 1/4). (b) C5b–7 site assay b: sheep (● 1/100); human (■ 1/30,000); rat (▲ 1/100); swine (▼ 1/10,000); goat (○ 1/100); rabbit (□ 1/1000); dog (△ 1/3000). Each point represents the mean of duplicate determinations (±SD).

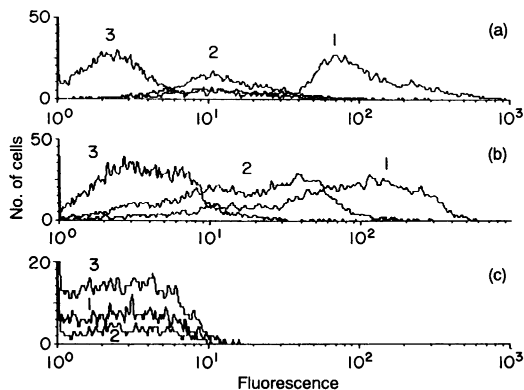

Figure 6. Flow cytometric analysis of SCIP on sheep blood cells. Sheep erythrocytes (a), lymphocytes (b) and platelets (c) were stained for SCIP with mAb S75 (1), pretreated with PIPLC and stained for SCIP (2) or incubated with non-immune mouse IgG as a control (3).

be used since CVF activation of sheep serum or a reactive lysis system utilizing human C5b6 and sheep EDTA serum did not give rise to lysis of the ShE in the presence or absence of S75. Monoclonal antibody S75 titrated against sensitized ShE resulted in a dose-dependent enhancement of lysis (Fig. 5). S75 titrated against unsensitized ShE did not give rise to lysis at any of the serum concentrations used, indicating that it was not itself C fixing.

The effect of S75 on lysis of ShE bearing preformed human C5b-7 sites (ShEC5b-7) was similarly investigated. Monoclonal antibody S75 again caused a dose-dependent enhancement of lysis of ShEC5b-7 upon subsequent addition of EDTA sheep serum as a source of C8 and C9 (Fig. 5).

Cellular distribution

The distribution of SCIP on sheep blood cells was examined by flow cytometry. More than 80% of erythrocytes and lymphocytes were positive after staining with mAb S75 compared to cells incubated with non-immune mouse IgG, but platelets were negative. PIPLC treatment of the cells reduced the binding of mAb S75 by 38% on erythrocytes and 35% on lymphocytes (Fig. 6).

Species specificity

The inhibitory activity of SCIP with respect to complement of seven different species was studied in two different activation assays. In the one assay GpE were incubated with SCIP, washed, and exposed to CVF and serum from different species (Fig. 7a). In the other assay, C5b–7 sites were first formed on GpE using CVF and C8-depleted rat or human serum, the cells were then incubated with SCIP, washed, and exposed to serum from different species as a source of C8 and C9 (Fig. 7b, data shown for rat C5b–7 sites only). In order to be able to observe effects of the inhibitor serum concentrations were chosen to obtain between 30 and 70% lysis of GpE or GpEC5b-7 without SCIP. The concentration of serum required to obtain 30–70% lysis varied enormously between the different species (see Discussion). As shown in Fig. 7, SCIP inhibited lysis by all sera tested in both assays.

Amino terminal protein sequence

An unambiguous sequence was followed for 27 cycles of Edman degradation. This, together with the amino terminal amino acid sequences of human CD59[5] and its rat homologue,[10] is shown in Fig. 8. In comparison with these other proteins a single amino acid deletion had occurred in the sheep inhibitor between amino acids 8 and 12 of the human sequence. No residues were released at cycles 3, 6, 12, 17, 18, 20 and 25. Positions 3, 6, 12, 18 and 25 correspond with cysteine residues in the human sequence. Reduction and alkylation of SCIP was not performed prior to sequence analysis and the lack of a signal at these positions is

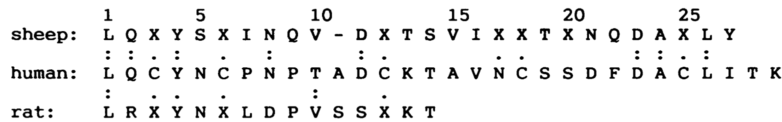

Figure 8. Amino terminal sequence of SCIP (Ov-CD59) aligned with the sequences for human and rat CD59. Residue numbering is for SCIP. A one residue insertion in the SCIP sequence is required for alignment. Residues conserved between SCIP and either human or rat CD59 are marked (:) and residues tentatively assigned as cysteine or aparagine as (.).

therefore compatible with these being cysteine residues in SCIP also. Position 17 aligns with asparagine 18 in the human sequence. In CD59 this asparagine is glycosylated. Similar glycosylation of an asparagine at position 17 in SCIP would account for lack of a signal at this position; threonine at position 19 is consistent with this hypothesis.

DISCUSSION

An inhibitor of complement-mediated lysis was isolated from membranes of sheep erythrocytes (SCIP) which bore close structural and functional resemblance to human CD59. A multi-stage FPLC procedure yielded a pure protein with an apparent M_r of 19,000 (Fig. 1a) but yields were very low (Table 1). Monoclonal antibody S75, raised against the FPLC-purified inhibitor facilitated the efficient purification of SCIP (Fig. 1c; Table 1). A pure protein was obtained when Lubrol-PX or NP-40 were used as detergents during the purification whereas using CHAPS, many other proteins coeluted with SCIP from the affinity column (Fig. 1c). This phenomenon is of interest in view of the recent findings of Stefanova and Horejsi[18] who have shown that human CD59 is tightly but non-covalently associated with other membrane components. Our findings suggest that SCIP may form similar associations and that the detergent chosen for extraction may influence these non-covalent interactions.

Monoclonal antibody S75 raised against SCIP on erythrocytes was shown to block the functional activity of this inhibitor, rendering ShE susceptible to lysis by sheep C (Fig. 5). The antibody also efficiently enhanced lysis when added to ShE after formation of C5b–7 sites indicating that SCIP acts at the final stages of MAC assembly.

Purified SCIP incorporated efficiently into GpE, rendering the cells resistant to lysis by sheep C, the degree of resistance being proportional to the amount of SCIP added (Fig. 2). SCIP inhibited lysis after the stage of C5b–7 site formation (Fig. 7b). These features suggest that SCIP is the functional equivalent of human CD59.[2–7] SCIP, incorporated into GpE, inhibited complement of all species tested. In this respect it differs from human CD59, which has been shown to inhibit efficiently lysis by human and primate C but is much less effective against C from other species,[3,5,11–13] but resembles the rat analogue of CD59 which is also species non-selective.[10] In these experiments, in order to obtain similar degrees of lysis in the absence of SCIP, it was necessary to use different sera at very different concentrations in both the CVF-mediated 'reactive lysis' system and on EC5b–7. This was necessary in order to observe inhibitory effects of the incorporated SCIP and defensible in that the requirements for different serum concentrations presumably reflect events prior to the site of action of SCIP such as the efficiency of activation by CVF or the efficiency of C8 recruitment by rat or human C5b–7.

The ability of SCIP to inhibit efficiently human C provides an explanation for the observation that incorporation of human CD59 into ShE does not protect from lysis by human C,[6] addition of a small amount of exogenous inhibitor having negligible effects in the presence of large amounts of endogenous inhibitor. We have used GpE as targets throughout these studies and it is possible that the lack of species selectivity observed is, in part, a function of the target cell. We are currently investigating this possibility by examining whether SCIP protects other target cells to the same extent.

Lack of species selectivity in animal equivalents of CD59 may have important consequences in the field of xenotransplantation. The hyperacute stage of transplant rejection is C mediated and may involve the MAC.[19] Choosing a species for organ donation, the endogenous CD59 of which efficiently inhibits human C, may therefore enhance graft survival. On the other hand, efforts to express human CD59 in transgenic animals with the aim of improving their utility as organ donors may be wasted if that species possesses a species non-selective CD59 analogue.

Amino terminal sequence of SCIP was obtained from the affinity-purified protein and compared with human and rat CD59 (Fig. 8). Assuming that the asparagine and cysteine residues are as predicted, 52% identity between the first 27 amino acids of human CD59 and SCIP and 40% identity between the first 15 amino acids of SCIP and rat CD59 were found. Even in the absence of these assignments it seems reasonable to conclude that SCIP is the structural, as well as the functional, equivalent of human and rat CD59. This is supported by other structural similarities of SCIP with human CD59, including the glycosylation pattern and GPI anchoring to the membrane. The apparent M_r of SCIP was reduced by 1000 and 4000 after treatment with neuraminidase and Endo-F respectively but *O*-deglycosidase did not alter the M_r. This suggests that SCIP, like human CD59, contains only N-linked carbohydrate.[3,5,11,12] Loss of functional activity of SCIP after Endo-F treatment indicates that the N-linked carbohydrate chain is important for the functional activity of this MAC-inhibiting protein. No data on functional activity of human or rat CD59 after deglycosylation have been reported. PIPLC treatment of SCIP resulted in the loss of inhibitory activity but did not affect the apparent M_r. Human erythrocyte CD59 is relatively resistant to PIPLC treatment due to modifications in its GPI anchor,[7,20] whereas rat erythrocyte CD59, like SCIP, was sensitive to PIPLC.[10] Deglycosylation did not reduce incorporation of SCIP into GpE whereas PIPLC treatment reduced inhibitory activity and binding to the same extent (Table 2). Monoclonal antibody S75 recognized the deglycosylated and the PIPLC-treated protein, although the latter appeared to stain less strongly both with silver on gels and with antibody on blots (Fig. 5). This observation is probably an experimental artefact caused by reduced efficiency of fixation and/or electrotransfer of the PIPLC-treated protein.

The cellular distribution of SCIP on blood cells was studied by flow cytometry. Erythrocytes and lymphocytes, but not platelets, were found to express SCIP (Fig. 6). The efficient removal of SCIP from both cell types by PIPLC treatment indicates that SCIP is inserted in the membrane through a simple phosphatidylinositol anchor. This is in contrast to human CD59 on erythrocytes, where the GPI anchor contains an extra lipid group and is therefore resistant to cleavage by PIPLC.[3,21] In contrast to human CD59, which is expressed and functionally active on platelets,[22,23] SCIP was not detectable on sheep platelets. It is possible that SCIP is not present on this cell type, but more likely that the mAb, raised against the inhibitor on sheep erythrocytes, does not recognize the platelet protein due to differences in glycosylation, or to steric hindrance by other cell surface proteins.

A potential equivalent of human CD59 in sheep has previously been described by Hein *et al.*[24,25] A mAb B5–5, raised against thymocytes, recognized an 11,000–17,000 MW GPI-anchored protein named B5 on thymocytes, T cells and B cells. However, the protein was not purified and no MAC inhibitory activity or amino terminal sequence data were described. Cross-linking of B5 through mAb B5–5 resulted in activation of peripheral T lymphocytes, prompting the suggestion that this was the sheep CD59 equivalent. Deglycosylation of B5 on lymphocytes with Endo-F reduced its M_r from 11,000–17,000 to 6000; these differences in M_r and size of the carbohydrate chain of SCIP and B5 made it unlikely that these are identical molecules. We have recently obtained a sample of the B5–5 antibody (kind gift of Dr Wayne Hein, Basel Institute for Immunology, Switzerland) and have confirmed that this antibody does not recognize SCIP. In the 'First International Workshop on Leukocyte antigens in cattle, sheep and goats'[26] the prefix 'Ov' was suggested for sheep (=ovine) equivalents of human CD antigens. The sheep inhibitor of complement, in this paper described as SCIP, shows all the structural and most of the functional characteristics of human CD59 and should thus be named Ov-CD59.

In summary, the sheep equivalent of human CD59, SCIP/Ov-CD59 was isolated and functionally characterized as a species non-selective inhibitor of C8/C9-mediated lysis. It is possible that CD59 equivalents from other species are similarly non-selective, a possibility with important consequences to xenotransplantation. Comparative analyses of CD59 analogues from different species should enable residues essential for the inhibitory activity of this important regulator to be identified.

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