Structural composition and functional characterization of soluble CD59: heterogeneity of the oligosaccharide and glycosphosphoinositol (GPI) anchor revealed by laser-desorption mass spectrometric analysis

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CD59 (protectin) is a glycosphosphoinositol (GPI)-anchored inhibitor of the membrane attack complex of complement found on blood cells, endothelia and epithelial cells. In addition to the lipid-tailed CD59, soluble lipid-free forms of CD59 are present in human body fluids. We have investigated the detailed structural composition of the naturally occurring soluble urinary CD59 (CD59_u) using peptide mapping, anion-exchange chromatography, sequential exoglycosidase digestion and matrix-assisted laser-desorption mass spectrometry (MALDI-MS). CD59_u exhibited an average M_r of 12444 in MALDI-MS. Mass analysis of the isolated C-terminal peptide (T9) indicated that a GPI-anchor (at Asn-77) without an inositol-associated phospholipid was present in soluble CD59_u. By using residue-specific exoglycosidases, chemical modification and MALDI-MS structures of seven different GPI-anchor variants were determined. Variant forms of the anchor had deletions and/or extensions of one or more monosaccharide units. Sialic acid linked to an *N*-

acetylhexosamine-galactose arm was found in two GPI-anchor variants. The N-linked carbohydrate side chain of CD59_u (at Asn-18) also displayed considerable heterogeneity. The predominant oligosaccharide chains were fucosylated biantennary and triantennary complexes with variable sialylation. Mono Q anion-exchange chromatography resolved urinary CD59 into nine different fractions that bound equally well to the terminal complement SC5b-8 complexes. Despite binding to C5b-8, soluble CD59_u inhibited complement lysis at an approx. 200-fold lower efficiency than erythrocyte CD59. These results document the structural heterogeneity of both the GPI anchor and N-linked oligosaccharide of CD59 and demonstrate that the phospholipid tail is needed for the full functional activity of CD59. The site of cleavage between the diradylglycerol phosphate and inositol suggests that a mammalian phospholipase D could be involved in the solubilization of GPI-anchored proteins.

INTRODUCTION

Activation of the plasma complement (C) system leads to formation of a cytolytic membrane attack complex (MAC) composed of the terminal C components C5b, C6, C7, C8 and multiple copies of C9 (reviewed in ref. [1]). While the main purpose of MAC is to destroy invading micro-organisms, the cells of the host have to be protected against self-destruction. Damaging effects of MAC are controlled by regulatory molecules which act either in the fluid phase or on the cell membrane. The human plasma protein, S-protein (vitronectin), is a multifunctional protein that acts as an acceptor for the aggregating late complement components and keeps them as a soluble non-cytolytic terminal complement complex (TCC) [2]. S-protein binds to the C5b67 complex and inhibits C9 polymerization by SC5b-8 [3]. Another body fluid protein, clusterin (SP40,40), binds to the TCC and appears to be capable of keeping it soluble in a manner similar to S-protein [4,5].

The CD59 antigen (MACIF, MIRL, HRF-20 or protectin) is a major inhibitor of the MAC present on human cell membranes [6–10]. CD59 inhibits complement lysis by preventing C5b-8-catalysed insertion and polymerization of C9 into cell membranes

[11,12]. The homologous restriction factor (HRF, C8bp, MIP) is another proposed inhibitor of MAC that binds to C5b-8 [13–15]. CD59 and HRF are anchored to cell membranes via a glycosphosphoinositol (GPI) anchor. Soluble forms of HRF have been found in human urine, plasma and cerebrospinal fluid, where they appear to exert a limited complement inhibitory function [15,16]. A soluble form of CD59 was initially reported in human urine [7]. CD59 is strongly expressed in the epithelia of many secretory organs [17] and has been detected in several body fluids [18–22a]. The presence of a lipid tail has been demonstrated for CD59 in amniotic fluid [18], seminal plasma [19] and breast milk [22]. In blood plasma CD59 has been detected in association with lipoprotein particles [23]. Recently, we observed that both soluble and lipid-tailed forms of CD59 are present in human urine [24].

The three-dimensional structure of soluble urinary CD59 [25] or of a recombinant CD59 composed of amino acid residues 1–70 [26] has recently been determined by two-dimensional NMR spectroscopy. The overall folding of CD59 resembles that of snake venom neurotoxins [27]. The disulphide bonding pattern of the ten cysteines of CD59 [26,28] defines a distinct domain that is found, for example, in the Ly-6 family of putative T-cell activation antigens [29] and in triplicate in the urokinase-type

Abbreviations used: MAC, membrane attack complex of complement; MALDI-MS, matrix-assisted laser desorption mass spectrometry; RP-HPLC, reverse-phase HPLC; GPI, glycosphosphoinositol; HRF, homologous restriction factor; CD59_e, CD59_u, erythrocyte and urinary forms of CD59; DAF, decay accelerating factor; GPE, guinea pig erythrocytes; PNGase F, peptide N-glycosidase F; TCC, terminal complement complex; uPAR, receptor for urokinase-type plasminogen activator; NHS, normal human serum; TFA, trifluoroacetic acid; ACH, α -cyano-4-hydroxycinnamic acid.

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plasminogen activator receptor (uPAR, CD87) [30]. The CD59 domain has two antiparallel β -sheets, one with three strands and another with two, that create a disc-shaped structure with extending loops [25,26].

The GPI anchor of CD59 is attached to the C-terminal Asn-77 residue and links the CD59 polypeptide to phospholipid. Initial biochemical analysis has indicated that the GPI-anchor structure resembles that of Thy-1 [31] in having a core structure of ethanolamine- PO_4 -($\pm$ Man α 1-2)Man α 1-2Man α 1-6Man α (PO_4 -ethanolamine; $\pm$ GalNAc)-GlcNH $_2$ α 1-6myo-inositol [32]. Using sequential exoglycosidase digestion Nakano et al. [32] have also examined the Asn-18-linked oligosaccharide of CD59 and report a biantennary complex-type structure. Detailed structural analysis of the oligosaccharide is warranted because glycosylation of CD59 has been suggested to be important for its complement lysis-inhibiting [33] and putative T-cell co-stimulating or signalling activities [34,35].

In the present study we have characterized in detail structural and functional properties of soluble urinary CD59 (CD59_u). CD59_u was carboxyamidomethylated, digested with trypsin, and the resulting peptides separated by reverse-phase HPLC (RP-HPLC). The N-linked glycopeptide and GPI-anchor peptide conjugates were hence isolated from each other to allow separate structural analysis using residue-specific exoglycosidases, chemical modification and matrix-assisted laser desorption mass spectrometry (MALDI-MS).

The results reveal a whole spectrum of structures of both the oligosaccharide linked to Asn-18 and of the GPI anchor (at Asn-77). The latter include previously undescribed variants containing sialic acid. The fact that the GPI anchor of CD59_u does not have an inositol-associated phospholipid suggests that mammalian phospholipase D could solubilize GPI-anchored glycoproteins that have become shed from cell surfaces. The soluble isoforms of CD59 retain their specific binding activity towards the TCCs but, because of the absent phospholipid tail, they only have a limited ability to inhibit MAC assembly on cell membranes.

MATERIALS AND METHODS

Antibodies and reagents

Rat hybridoma cells producing an anti-CD59 mAb (YTH53.1; kindly obtained from Professor H. Waldmann, Department of Pathology, University of Cambridge, Cambridge, U.K.) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) IgG-free fetal calf serum (Gibco). The IgG fraction was collected from the culture supernatants by protein G-Sepharose chromatography (Pharmacia Biotechnology, Uppsala, Sweden). Mouse anti-CD59 (BRIC 229) monoclonal antibody was from International Blood Group Reference Laboratory (Bristol, U.K.). The following supplies were purchased as indicated: Lubrol PX, Triton X-100, Nonidet P-40, n-octyl glucoside, EDTA, hydrofluoric acid, inulin, trypsin (from bovine pancreas), *Arthrobacter ureafaciens* sialidase (Sigma Chemical Co., St. Louis, MO, U.S.A.); endoproteinase Lys-C (Boehringer Mannheim, Mannheim, Germany), *Flavobacterium meningosepticum* peptide N-glycosidase F (PNGase F), *Streptococcus pneumoniae* galactosidase, galactosidase from bovine testes, *S. pneumoniae* N-acetylglucosamidase, chicken liver N-acetylglucosamidase, jack bean α -mannosidase, *Helix pomatia* mannosidase, jack bean N-acetylhexosaminidase (Oxford Glycosystems, Oxon., U.K.). Sera depleted of the terminal complement components (C7DS, C8DS and C9DS) were a kind gift from Dr. B. P. Morgan, University of Wales College of Medicine, Cardiff, U.K. C56 complexes were isolated from a pool of acute-phase

sera by euglobulin precipitation, gel-filtration and anion-exchange chromatography on a DEAE-Sephadex column (Pharmacia Biotechnology). The purity of C56 was examined by SDS/PAGE and activity titrated in a reactive lysis system using normal human serum (NHS)-EDTA and guinea pig erythrocytes (GPE).

Isolation of soluble and membrane forms of CD59

The erythrocyte membrane (CD59_e) and urinary forms of CD59 were purified by YTH53.1-Sepharose 4B affinity chromatography [7,11]. To obtain soluble urinary CD59 (CD59_u), the possibly remaining lipid-tailed CD59 associated with small micelles or vesicles in the urinary CD59 preparation was removed by centrifugation at 100 000 g for 1 h followed by gel filtration on Superose 6 (Pharmacia Biotechnology) [24]. Proteins were radiolabelled with ¹²⁵I using the Iodogen method (Pierce Chemicals, Rockford, IL, U.S.A.). The initial specific radioactivities of the radiolabelled proteins in representative samples were: CD59_e, 2×10^7 c.p.m./ μ g; and CD59_u, 1.3×10^7 c.p.m./ μ g respectively. As compared with unlabelled CD59_e the ¹²⁵I-labelled CD59_e retained 80% of its complement lysis inhibitory activity in a reactive lysis assay system [11].

Mono Q anion-exchange chromatography and SDS/PAGE

Affinity-purified urinary CD59 was chromatographed on an HPLC Mono Q column (HR 5/5, Pharmacia Biotechnology) equilibrated with 20 mM Tris/HCl, pH 7.2, and eluted with a linear gradient of 0 to 200 mM NaCl in the equilibration buffer. The eluting proteins were monitored on-line by absorbance at 218 nm. SDS/PAGE was performed according to Laemmli [36] using 12% or 15% Protean II minigels (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Enzymic digestions and amino acid sequence analysis

Urinary CD59 was subjected to *A. ureafaciens* sialidase, 1.0 IU/ml, treatment in 50 mM Tris/HCl, pH 5.5, at 37 °C overnight, diluted with 2 vols. of Milli-Q water and applied on to the Mono Q column. PNGase F digestion of CD59_u (for PNGase F digestion of N-linked glycopeptide see Table 1) was carried out in 50 mM potassium phosphate buffer, pH 6.1, containing 1% n-octyl glucoside, 0.1% SDS and 5 mM EDTA at 4 °C for 16 h (in the presence or absence of β -mercaptoethanol). Endoproteinase Lys-C and trypsin digestions were carried out in 1% NH₄HCO₃ solution. Purified CD59 (CD59_e or CD59_u) was incubated with 1.5% (w/w) of enzyme for 2 h at 37 °C followed by addition of another 1.5% (w/w) of the same enzyme. The incubation was continued at 37 °C for 2–4 h. The released peptides were separated by RP-HPLC. Sequence analysis was performed by using automated Edman degradation and a modified Applied Biosystems 477A/120A sequencer in the pulsed liquid mode [37].

Prior to digestion with trypsin CD59_u was carboxyamidomethylated as follows: CD59_u (113 μ g in 200 μ l of one-tenth diluted PBS) was lyophilized without desalting and resuspended in 25 μ l of 8 M urea, 100 mM ammonium bicarbonate (prepared fresh and deionized on a mixed-bed resin). Dithiothreitol (1 μ l, 450 mM) was added and the mixture incubated at 50 °C for 15 min. Carboxyamidomethylation was performed using 10 μ l of iodoacetamide (100 mM) and incubated at room temperature for 15 min. Water (64 μ l) was added to dilute the urea prior to trypsin digestion. Modified sequence-grade trypsin (Promega Corp., Madison, WI, U.S.A.) was added (5 μ l of a

Table 1 Specificities and compositions of glycosidase mixturesAbbreviations: GlcNAcase, β -N-acetylglucosaminidase; PNGase F, peptide N-glycosidase F; HexNAcase, N-acetylhexosaminidase.

(a) N-linked glycopeptides

Enzyme	Source	Linkage specificity*	Concn. (unit/ml)	pH optimum	PNGase F digest	Exoglycosidase digests (volumes in μ l)							
						1	2	3	4	5	6	7	8
PNGase F	<i>Flavobacterium meningosepticum</i>		20	7.0–8.0	0.4								
Sialidase	<i>Arthrobacter ureafaciens</i>	α 2-6 > 3,8	1	5.0–5.5		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Galactosidase	<i>Streptococcus pneumoniae</i>	β 1-4	0.2	5.5–6.5			0.1	0.1	0.1	0.1	0.1	0.1	0.1
GlcNAcase	<i>Streptococcus pneumoniae</i>	β 1-2	0.004	5.0–5.5				0.1			0.1		
GlcNAcase	Chicken liver	β 1-2,3,4,6	1	4.0–4.5					0.1	0.1		0.1	0.1
Mannosidase	Jack bean in 10 mM ZnCl ₂	α 1-3	0.1	4.0–4.5						0.1			
Mannosidase	Jack bean in 10 mM ZnCl ₂	α 1-2,3,6	10	4.0–4.5							0.1	0.1	0.1
Mannosidase	<i>Helix pomatia</i>	β 1-4	1	4.0–4.5									0.1
HPLC fraction/sample					0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Buffer A†					0.1								
Buffer B‡						0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
10% Methanol						0.3	0.45	0.35	0.35	0.15	0.25	0.25	0.15

(b) GPI anchor peptide conjugates

Enzyme	Source	Linkage specificity*	Concn. (unit/ml)	pH optimum	Reactions (volumes in μ l)				
					1	2	3	4	5
Sialidase	<i>Arthrobacter ureafaciens</i>	α 2-6 > 3,8	1	5.0–5.5	0.1				
Galactosidase	Bovine testes	β 1-3,4 > 6	1	4.0–5.0		0.2			
HexNAcase	Jack bean	β 1-2,3,4,6	3.4	6.0–8.5			0.2		
Mannosidase	Jack bean in 10 mM ZnCl ₂	α 1-2,3,6	10	4.0–4.5				0.1	
48% Hydrofluoric acid									0.5
HPLC fraction					0.25	0.25	0.25	0.25	0.25
Buffer B†					0.1	0.1	0.1	0.1	
10% Methanol					0.55	0.45	0.45	0.55	0.25

* Specificity data were taken from Oxford Glycosystems Ltd. data sheets and product information.

† Buffer A = 20 mM ammonium bicarbonate, pH 8.0, 50% methanol.

‡ Buffer B = 50 mM sodium citrate/phosphate, pH 5.0.

2 mg/ml solution) and incubated at 28 °C for 24 h. The final protein to protease ratio was 11.3:1 (w:w).

Prior to digestion with endoproteinase Lys-C (Wako Chemicals, CA, U.S.A.) CD59_U was vinylpyridylated as follows: 10 μ g of the protein was incubated with the alkylation buffer (6 M guanidium hydrochloride, 0.5 M Tris/HCl, 2 mM EDTA) and reduced with 5 μ l of dithiothreitol at 22 °C for 10 min. After incubation 1 μ l of 4-vinylpyridine (Aldrich Chemicals, Steinheim, Germany) was added and the mixture was incubated at 22 °C for 10 min. The reduced and alkylated protein was desalted by HPLC and subjected to digestion (2 $\times$ 2 h at 37 °C) with endoproteinase Lys-C (1.5 %).

Digested CD59_U (8.8 nmol in 200 μ l) was applied to a Brownlee RP-300 column (C8; 100 mm $\times$ 2.1 mm). Peptides were separated by RP-HPLC on an Applied Biosystems 130A System with a linear gradient of acetonitrile [solvent A = 0.1 % trifluoroacetic acid (TFA); solvent B = 0.085 % TFA in acetonitrile; flow rate, 100 μ l/min] over a 100 min cycle. Peptide peaks were detected at a wavelength of 220 nm, and collected, lyophilized and stored at –20 °C until required for analysis.

Glycosidase digestion of T2-glycopeptide and T9-GPI-anchor peptides

Digestions were performed in a total volume of 1 μ l (see Tables

1a and 1b for digestions of the N-linked oligosaccharide and the GPI anchor, respectively) [38]. After the final addition to each digest, the tubes were vortexed briefly and centrifuged at 18000 g for 1 min. The incubations were performed at room temperature and aliquots removed after 18–24 h. Each aliquot was used immediately to prepare sample slides for MALDI-MS.

MALDI-MS analysis

The mass spectrometer used in this work was a Finnigan LASERMAT 2000 (Finnigan MAT Ltd., Hemel Hempstead, U.K.) using a pulsed nitrogen laser (337 nm), 0.5 m drift tube and multi-channel plate detector. The detector signal was digitized at a sampling rate of 100 MHz and transferred to a personal computer for storage and report generation.

Native CD59_U and sialidase-treated CD59_U were analysed in 0.5 μ l of α -cyano-4-hydroxycinnamic acid (ACH) at 10 mg/ml in acetonitrile/water (70:30, v/v), in the absence of an internal calibrant. HPLC fractions were analysed in the presence of an internal calibrant (substance P, $[M+H]^+ = M_r$ 1348.7), T2 N-glycosylation exoglycosidase digests in the presence of PNGase F-treated (deglycosylated) T2 ($[M+H]^+ = M_r$ 1804) and T9-GPI digests in the presence of CD59 T5 peptide ($[M+H]^+ = M_r$ 1540.6). ACH (0.5 μ l at 10 mg/ml in acetonitrile/water (70:30, v/v) was used as a matrix throughout.

Cell binding and haemolysis assays

Binding of soluble and membrane forms of CD59 to GPE was analysed by mixing serial dilutions of radiolabelled proteins with GPE (2×10^7 cells/ml) and incubating them for 60 min at 37 °C. Proteins were diluted in PBS in the presence (CD59_E) or absence (CD59_U) of 0.001 % Lubrol PX detergent. Radiolabelled sheep IgG was used as a control for non-specific binding. After washing with PBS, the cells were counted for bound radioactivity.

In the reactive haemolysis assay 10^7 GPE were mixed with C56 euglobulin and increasing concentrations of CD59_E or CD59_U. Cells were incubated with EDTA plasma (25 %) for 30 min at 37 °C and haemolysis was quantified as release of haemoglobin (A_{412}). All lysis experiments were performed in duplicate.

Sucrose density-gradient ultracentrifugation

Binding of CD59 to TCCs was investigated by incubating (60 min, 37 °C) 125 I-CD59_U (50 ng) with 60 μ l of C7DS, C8DS, C9DS or NHS in the presence or absence of 4 % (w/v) inulin [39]. Bound radioactivity was separated from unbound ligand by sucrose density ultracentrifugation (10–50 % gradients in PBS, centrifugation for 16 h at 40 000 *g*) and binding was calculated as percentage of label incorporated in the high-molecular-mass complexes.

RESULTS

Comparison of binding properties of soluble and lipid-tailed CD59

Earlier studies have demonstrated the presence of both soluble and lipid-tailed forms of CD59 in various human body fluids including sweat, tears, saliva, breast milk, seminal plasma, amniotic fluid and blood plasma [18,19,22,22a,23]. In the present study soluble urinary CD59 (CD59_U) was chosen for closer structural and functional studies. CD59 was isolated from human urine by YTH53.1 monoclonal antibody affinity chromatography. N-terminal sequencing of urinary CD59 yielded the sequence LQCYNCPNPTADCK, which is identical to the N-terminal sequence derived from the cDNA sequence of CD59 [7]. When freshly isolated urinary CD59 was radiolabelled and subjected to a Triton X-114 phase separation procedure, approx. 2.4 % of the radiolabel was observed to partition with the detergent phase. In a separate study [24] employing Triton X-114 phase separation and a specific ELISA to quantify CD59 we observed that 90.1 % and 9.1 % of CD59 in a urine pool from healthy donors partitioned into the aqueous and detergent phases, respectively.

The glycolipid-anchored CD59_E has previously been shown to be capable of inserting into erythrocyte membranes [11]. In contrast with 125 I-CD59_E, soluble 125 I-CD59_U did not enter the cell membrane when mixed with human or guinea pig erythrocytes (Figure 1a). On a molar basis the binding of 125 I-CD59_U to red blood cells did not exceed that of the soluble radiolabelled control protein (sheep IgG).

Functional activity of soluble CD59: inhibition of complement lysis and binding to TCCs

The MAC-inhibiting activities of CD59_E and CD59_U were compared in a reactive lysis system. As shown in Figure 1(b), CD59_U inhibited C56 + C7 + C8 + C9-induced haemolysis of GPE. Approx. 1 μ M of CD59_U versus 5 nM of CD59_E was required for 50 % inhibition of haemolysis under the conditions used.

CD59_E has previously been shown to interact with the terminal C5b–8 and C5b–9 complexes [11]. To see if soluble CD59_U had

Figure 1 Functional comparison of soluble CD59_U and lipid-tailed CD59_E.

(a) Binding of CD59_U and CD59_E to GPE membranes. Various amounts of radiolabelled CD59_E (2×10^7 c.p.m./ μ g, in PBS with 0.001 % Lubrol PX), CD59_U (1.3×10^7 c.p.m./ μ g) or sheep IgG (1.2×10^7 c.p.m./ μ g; control) were mixed with 2×10^7 GPE in PBS and incubated for 60 min at 37 °C. Bound radioactivity was determined after centrifugation of cells through 20 % sucrose. (b) Inhibition of complement lysis by soluble and membrane forms of CD59. 10^7 GPE were mixed with C5b6 and increasing concentrations of CD59_E or CD59_U. Lysis was developed with EDTA plasma (25 %) and quantified as release of haemoglobin (A_{412}). CD59_U inhibits lysis of GPE at an approx. 200-fold lower efficiency than CD59_E.

equivalent activities, fluid-phase TCCs SC5b67, SC5b–8 and SC5b–9 were prepared by activating sera depleted of C8, C9 or NHS with 4 % inulin (60 min, 37 °C). 125 I-CD59_U was added to the sera prior to activation. In line with results obtained earlier [39] approx. 42 % and 19 % of offered 125 I-CD59_U bound to the SC5b–8 and SC5b–9 complexes, respectively. No binding to the C5b6 or SC5b67 complexes or to any high-molecular-mass complexes in zymosan-activated rabbit serum occurred.

Molecular heterogeneity of urinary CD59

When affinity-purified urinary CD59 was applied on a Mono Q strong anion-exchange chromatography column it was found to resolve into nine distinct peaks, which in SDS/PAGE showed heterogeneity in the apparent molecular mass (Figure 2). When the same sample of urinary CD59 was treated with *A. ureafaciens* sialidase prior to Mono Q chromatography the molecular heterogeneity was observed to diminish and a novel peak in the

Figure 2 Heterogeneity of urinary CD59 revealed by anion-exchange chromatography

(a) Affinity-purified urinary CD59 was applied on a Mono Q column equilibrated with 20 mM Tris/HCl, pH 7.2, and eluted with a linear gradient of NaCl. Elution was monitored by A_{218} (AU, absorbance units). (b) SDS/PAGE analysis (silver staining) shows fractions 1, 2, 3, 5, 6 and 9 run under reducing conditions. t, time.

Mono Q breakthrough fraction appeared (Figure 3a). The apparent molecular mass of sialidase-treated urinary CD59 in SDS/PAGE was only slightly smaller than that of untreated CD59_U (Figure 4b). Examination of the individual radiolabelled fractions from Mono Q separation, including fraction N1 after sialidase treatment, showed that each fraction had a quantitatively similar capacity to bind to the SC5b-8 complexes generated in inulin-activated C9DS (Table 2 and Figure 3c).

In line with earlier results with CD59_B [6] the treatment of CD59_U with PNGase F reduced its apparent molecular mass by approx. 6 kDa in SDS/PAGE (Figure 4c). Removal of the N-linked carbohydrate was successful only in the presence of a reducing agent. This prevented the re-analysis of the molecule by RP-HPLC (but not MALDI-MS). On the other hand, PNGase F was not able to cleave the non-reduced molecule as shown in the inset of Figure 4(c). Therefore only the isolated and repurified tryptic peptide (T2) has been cleaved with PNGase F. Examination of the effect of N-deglycosylation on the functional activity of CD59_U was not possible in this approach, since reduction of disulphides in CD59_U rendered it incapable of binding to SC5b-8.

Peptide mapping and mass spectrometric analysis of CD59_U

Purified soluble CD59_U was reduced, alkylated and digested with endoproteinase Lys-C and trypsin into peptides that were separated by RP-HPLC (Figure 5). The isolated peptides were subjected to automated Edman degradation and amino acid sequence analysis. All peptide sequences obtained matched the reported amino acid sequence of CD59 [7]. Only one amino acid, Asn-18, could not be identified due to the glycosylation of this residue [33]. The C-terminal amino acid was confirmed as Asn-77 by automated N-terminal sequence analysis of the isolated C-terminal peptide T9.

MALDI-MS analysis of native soluble CD59_U gave two major broad mass signals with average M_r values of 12444 and 11164 (Figure 4a). The spectrum showed additional lower-molecular-mass peaks which may be due to $[M + 2H]^+$ ions. After treatment of CD59 with *Arthrobacter ureafaciens* sialidase to remove terminal sialic acid residues more clearly defined peaks for

CD59_U were observed (Figure 4b). The broad peaks were thus principally due to variable sialylation. The multiplicity of the peaks, however, indicated significant remaining heterogeneity in the CD59 preparation.

After treatment with PNGase F to remove the Asn-18-linked oligosaccharide, CD59_U still exhibited heterogeneity in its MALDI-MS spectrum (Figure 4c). As shown below, this was apparently due to heterogeneity in the GPI anchor, of which two major variants (C + C', D) linked to the CD59 polypeptide could be deduced from the mass values.

MALDI-MS analysis of N-linked oligosaccharides of CD59_U

RP-HPLC fractions of the separated CD59_U tryptic peptides were analysed by MALDI-MS. Three fractions exhibited complex spectra associated with glycosylation heterogeneity. Detailed inspection of the spectra revealed mass differences between adjacent signals corresponding to monosaccharide residues, e.g. M_r 162 corresponding to a hexose (glucose, mannose, or galactose), M_r 146 to fucose, M_r 203 to an N-acetylhexosamine (N-acetylglucosamine or N-acetylgalactosamine), or M_r 291 to N-acetylneuraminic acid (Figures 6A and 6B).

When treated with PNGase F (Table 1a), and re-analysed by MALDI-MS, the complex spectra of two of the fractions (one shown in Figure 6a) were replaced by a single mass of $MH^+ = 1803.7$ (Figure 7a). This signal corresponds to the carboxyamidomethylated T2 peptide (with Asn converted into Asp by PNGase F) containing the N-linked recognition sequence -TAVN¹⁸C'SSD (C' = carboxyamidomethylated cysteine). The third fraction (Figure 6B) was not affected by PNGase F treatment.

The two fractions containing the N-linked glycopeptide were combined and used for sequence analysis of the oligosaccharide structures as described by Sutton et al. [38]. The calculated mass value of the free T2 peptide (M_r 1803.0) was subtracted from each of the mass values in the native glycopeptide spectrum to give the residue masses of the oligosaccharide mass. Using the residue masses of monosaccharides that occur naturally in mammalian glycoproteins it was possible to assign a probable composition to each glycoform and tentatively identify it as belonging to a particular class of oligosaccharides. The T2 oligosaccharide compositions indicated that fucosylated bi- and tri-antennary complexes predominated, with a smaller contribution from non-fucosylated bi- and tri-antennary complexes. To confirm these assignments and obtain more sequence information, the T2 glycopeptide was treated with specific exoglycosidases and subjected to MALDI-MS [38].

The T2 glycopeptide fraction was split into eight aliquots and digested using the enzyme mixtures listed in Table 1(a). The digests were run simultaneously and then analysed by MALDI-MS (Figures 7b-7h). The MALDI-MS results from each digest have been interpreted in terms of progressive digestion of the oligosaccharide structures and represented as a flow chart (Figure 8). Because of the sugar and linkage specificity of the exoglycosidases the shift in masses with inclusion of each new enzyme enabled the sequencing of the oligosaccharides attached to the T2 peptide. Previous experiments [38] had shown that after sialic acid had been removed with *Arthrobacter ureafaciens* sialidase, the mass signal intensities of each glycoform attached to the peptide were proportional to site occupancy for each glycoform. Quantification was based on the intensity of each signal as a fraction of the integral intensity of all signals assigned to glycopeptides after sialidase digestion.

The data collected from treatment of the T2 glycopeptide with specific exoglycosidases, MALDI-MS analysis and quantification

Figure 3 The effect of sialidase on urinary CD59

(a) Mono Q profile of urinary CD59 after treatment with *A. ureafaciens* sialidase. Note appearance of fraction N1 and decrease in the other fractions. (b) SDS/PAGE analysis (silver staining) compares fractions N1–N3 with fractions 1 and 9 (from Figure 2). No protein in fraction N3 becomes visualized in the silver-stained SDS/PAGE gel. Because of its ability to incorporate into cell membranes and TCC the material in fraction N3 may represent a trace of a phospholipid-tailed form of CD59 with a disproportionately high absorbance at 218 nm. (c) Comparison between CD59_U (fraction 1 from Figure 2) and sialidase-treated CD59_U (fraction N1) for their ability to bind to SC5b–8. To generate fluid-phase SC5b–8 C9-depleted human serum was activated (60 min, 37 °C) with 4% inulin in the presence of ¹²⁵I-CD59_U (50 ng) and subjected to sucrose density-gradient analysis. IgG (7 S) and IgM (19 S) were used as migration markers. Top of the gradient is to the left. t, time.

from the sialidase-treated spectra enabled the structure of the glycoforms to be determined (Figure 9).

Characterization of the C-terminal GPI-anchor moiety of CD59_U

A single HPLC fraction gave a complex spectrum in the M_r range 2400–3400 (Figure 6B). When treated with 48% hydrofluoric acid (to cleave labile phosphate bonds that link the GPI anchor to the peptide), the putative T9-GPI-anchor signals (A to F) disappeared and were replaced by a signal of M_r 1440.3 (Figure 10A) which corresponded to the T9 peptide with a single ethanolamine group attached.

The most prominent signal, C (M_r 2817.4), of the HPLC fraction (Figure 6B) had a mass consistent with T9 peptide conjugated to a Thy-1-type GPI anchor, but lacking the phosphatidyl group. The HPLC fraction was split into several aliquots and treated with a range of exoglycosidases (Table 1b). Incubation with *S. pneumoniae* N-acetylglucosaminidase, chicken

Figure 4 MALDI-MS spectra of native CD59_U (a), sialidase- (b) and PNGase F- (c) treated CD59_U

(a) Approx. 0.4 pmol of soluble CD59_U was applied on a Lasermat 2000 mass spectrometer. The native spectrum gives two major and three minor peaks with indicated average mass values. The broad mass peaks are due to molecular mass heterogeneity of the sample. The identity of the two major peaks is based on mass calculations that consider structures of the N-linked oligosaccharide (CHO) and C-terminal GPI-anchor (see Figures 9 and 11). (b) MALDI-MS analysis of CD59_U after treatment with *A. ureafaciens* sialidase. The broad peaks of A are resolved into distinct molecular mass species. A small reduction in the apparent molecular mass of CD59_U after sialidase treatment can be seen in a silver-stained SDS/PAGE gel (inset). (c) On treatment with PNGase F the dominant peak of CD59_U still exhibits heterogeneity due to the GPI anchor. $[M+H]^+$ values indicate the calculated expected mass values of protonated products. The masses of the major forms of the GPI anchor (C, C', D) are from Figure 11. A Coomassie Blue-stained SDS/PAGE gel in the inset shows the effect of PNGase F on CD59_U in the absence or presence of a reducing agent.

Table 2 Incorporation of Mono Q fractions of ^{125}I -labelled urinary CD59 into SC5b-8 complexes

Binding is expressed as a percentage of specific incorporation of ^{125}I -labelled urinary CD59 fractions into the terminal SC5b-8 complex during incubation of C9DS with 4% inulin for 60 min at 37 °C.

Mono Q fraction number	Incorporation (%)
1	20.1
2	21.4
3	25.9
4	25.7
5	31.4
6	25.9
7	21.9
8	22.2
9	31.3

Figure 5 RP-HPLC profile of peptides from CD59_u after digestion with endoproteinase Lys-C

Approx. 10 μg of CD59_u was digested with endoproteinase Lys-C (1.5%) for 2×2 h at 37 °C and the resulting peptides were separated by RP-HPLC. Amino acid sequences of the selected peptides were determined by automated Edman degradation.

liver *N*-acetylglucosamidase or coffee bean α -galactosidase did not result in any mass changes of any of the signals (results not shown). Jack bean α -mannosidase, however, resulted in M_r changes of 162 for signals C, D and F, indicating the presence of an exposed mannose (Figure 10B). Bovine testis β -galactosidase reduced the signal intensity of C and resulted in the complete loss of D (Figure 10C). With both these enzymes there was a concomitant increase in signal B intensity.

The information from the digests was used to deduce possible structures of the GPI-anchor moiety (Figure 11). We have assumed that the Man-Man-Man (ethanolamine-phosphate)-glucosamine-inositol core is the same as that observed in the well-characterized Thy-1 GPI anchor [31] and that the position of the exposed sugar, cleaved by exoglycosidases, is analogous to that observed in the scrapie prion protein Prp^{Sc} [41]. Signal C comprises two possible structures containing either an exposed mannose (structure C) or a galactose (structure C') residue.

Figure 6 Mass analysis of RP-HPLC fractions containing CD59_u tryptic fragments

(A) Native T2 glycopeptide separated by RP-HPLC. Further analysis of the heterogeneous T2 glycopeptide is shown in Figure 7. (B) C-terminal T9-GPI-anchor peptide. Interpretation of signals A–F is illustrated in Figure 11. The 1540.6 mass peak corresponds to the T5 peptide that served as an internal standard for the mass analysis.

Signal B constitutes structure C without the exposed mannose residue and signal A lacks the mannose and a putative *N*-acetylhexosamine residue. In Thy-1, the latter residue is an exposed *N*-acetylglucosamine, but in CD59 the respective residue was not affected by jack bean *N*-acetylhexosaminidase (results not shown). It is possible that jack bean hexosaminidase does not recognize *N*-acetylhexosamine in the context of the GPI structure or that neither *N*-acetylglucosamine nor *N*-acetylglucosamine is present. Signal D was modified by both α -mannosidase and β -galactosidase, suggesting the presence of exposed residues on the same molecule (structure D). *A. urea-faciens* sialidase digestion resulted in loss of E and F signals, indicating the presence of exposed sialic acid residues (Figure 10D). Signal F was also lost on α -mannosidase digestion, indicating the presence of an exposed mannose residue on structure F.

For legend see opposite.

Figure 8 Composite glycoforms identified in each exoglycosidase digest of CD59_U tryptic fragment

Each structure was based on the mass signal shifts identified by MALDI-MS before and after digestion with a specific exoglycosidase in digests 1 to 8 of Table 1(a).

DISCUSSION

In addition to cell membranes the complement MAC-inhibitor CD59 (protectin) is present in human body fluids and secretions. As indicated in the present study a high proportion of CD59 in human urine represents a soluble, phospholipid-free isoform (CD59_U) that binds to the TCCs and has limited complement haemolysis-inhibiting activity. CD59_U was found to have an average M_r of 12 444, of which the main N-linked oligosaccharide accounts for $\approx 19\%$ and the GPI anchor for $\approx 11\%$ depending on the degree of their structural modifications.

The 77-amino-acid-long CD59 was found to express considerable heterogeneity both in its oligosaccharide side chain and in the C-terminal GPI anchor. Affinity-purified urinary CD59 resolved into at least nine different peaks in Mono Q chromatography (Figure 2a). As Mono Q is a strong anion-exchange column the various forms of urinary CD59 separated must have different charged groups exposed on their surfaces under the buffer conditions used. Mono Q fractionation of the sialidase-

treated urinary CD59 yielded a major breakthrough peak, indicating that the charge heterogeneity was due to differential sialylation of CD59. A similar result was obtained using MALDI-MS, where the diffuse major mass peak of CD59_U resolved into distinct peaks after sialidase treatment (Figure 4b).

Variable sialylation in the Asn-18 oligosaccharide of CD59 was directly demonstrated by MALDI-MS analysis of the T2-CHO peptide. Treatment of T2 with sialidase significantly simplified the spectrum and allowed assignment of core structures of the oligosaccharide (Figure 7). Heterogeneity in the oligosaccharide backbone was due to the presence of either bi- or tri-antennary complexes. Treatment of T2 with PNGase F abolished the heterogeneity by removing the oligosaccharide side chain from the peptide.

Our results differ from those of Nakano et al. [32] in that we detected a significant proportion (22%) of triantennary structures and a broader distribution of various oligosaccharide structures in CD59. In accordance with this study we observed that the most common oligosaccharide type is a biantennary complex with fucose linked to the proximal N-acetylglucosamine residue.

The sialylation heterogeneity of urinary CD59 was not found to significantly affect its binding activity towards the terminal SC5b-8 complex (Table 2). Thus, in accordance with observations made by Ninomiya et al. [33], it is unlikely that the degree of sialylation would affect the functional activity of CD59. On the other hand, the removal or chemical modification of the backbone of the N-linked sugar was suggested to lead to a substantial reduction in the complement lysis inhibitory activity, but not in the TCC binding activity of erythrocyte CD59 [33]. The mechanism of the possible contribution of the oligosaccharide to the MAC inhibitory function of CD59 remains to be established.

The presence of the GPI anchor is a characteristic feature of the complement regulators decay accelerating factor (DAF) and CD59, as well as of many non-complement molecules. Mass analysis of the GPI anchor on the C-terminal peptide of CD59_U yielded a main signal of M_r 2817.4, which is close to a theoretical protonated mass, M_r 2818.0, of structures C and C' in Figure 11. The principal putative GPI structure of CD59 (C in Figure 11) is similar to that of mammalian Thy-1 GPI anchor [31] without an inositol-associated phosphatidate group. The apparent absence of the diradylglycerolphosphate group from CD59_U could be due to enzymic cleavage of the GPI anchor *in vivo*. Phospholipase D in human plasma [40] is an enzyme capable of cleaving GPI anchors between inositol and phosphate. Solubilization of GPI-anchored molecules could thus provide a physiological function for plasma phospholipase D, for which the identity of natural substrates is still unknown. In order not to affect GPI-anchored molecules on cell membranes the detachment of GPI proteins from their lipid tails should occur only after the molecules have become shed from cell membranes. How this precisely occurs and whether any regulatory mechanisms are involved in this process remain to be investigated.

The mass signals for the T9-GPI-anchor peptide also showed significant heterogeneity in the M_r range 2400–3400 (Figure 6B).

Figure 7 Mass analysis of the T2 glycopeptide after digestion with glycosidases (Table 1a)

(a) PNGase F digestion, (b) digest 1, *Arthrobacter ureafaciens* sialidase, (c) digest 2, *A. ureafaciens* sialidase and *Streptococcus pneumoniae* galactosidase, (d) digest 3, *A. ureafaciens* sialidase, *Strept. pneumoniae* galactosidase and *Strept. pneumoniae* N-acetylglucosaminidase, (e) digest 4, *A. ureafaciens* sialidase, *Strept. pneumoniae* galactosidase and chicken liver N-acetylglucosaminidase, (f) digest 5, *A. ureafaciens* sialidase, *Strept. pneumoniae* galactosidase, chicken liver N-acetylglucosaminidase and jack bean α -mannosidase, (g) digest 6, *A. ureafaciens* sialidase, *Strept. pneumoniae* galactosidase, *Strept. pneumoniae* N-acetylglucosaminidase and jack bean α -mannosidase, (h) digest 8, *A. ureafaciens* sialidase, *Strept. pneumoniae* galactosidase, chicken liver N-acetylglucosaminidase, jack bean α -mannosidase and *Helix pomatia* β -mannosidase.

CD59 Asn₁₈ Oligosaccharide Structure

Core Oligosaccharides

Biantennary Complexes

Triantennary Complexes

Tetraantennary Complexes < 1 %

Figure 9 Structures of oligosaccharides linked to Asn-18 of soluble urinary CD59

The linkage information was determined by a combination of MALDI-MS analysis with sequential treatment of the desialylated T2-CHO peptide with linkage-specific exoglycosidases (Table 1a; Figures 7 and 8). The percentages indicate the approximate proportions of each structure determined from peak heights from the spectra of sialidase-treated T2-CHO glycopeptide (Figure 7b).

The different signals are due to structural modifications of the anchor structures. The two major forms (C and C') with the same mass value differ in having either an exposed mannose (C) or galactose residue. Further heterogeneity is due to the absence (B) or presence (D) of both of these residues, an absent *N*-acetylhexosamine (A) or the presence of a sialic acid residue (E, F). In the present study it was not possible to determine the exact locations of the sialic acid residue or of the *N*-acetylhexosamine-galactose-sialic acid arm. The presence of a trisaccharide arm on GPI anchors is a relatively novel observation and has previously been described only once before, on a scrapie prion protein [41].

In this study it was determined that the trisaccharide was linked to the third mannose from the peptide [41]. Therefore, an analogous putative position for the trisaccharide is shown in Figure 11.

Similarities in the GPI anchors of Thy-1 [31], human erythrocyte acetylcholine esterase [42], placental alkaline phosphatase [43] and CD59 [32] suggest a consensus structure for mammalian GPI anchors, which with slight modifications extends to that of single cell eukaryotes and protozoal parasites [44,45]. With the currently available glycosidases it was not possible to determine the sugar linkages of the core GPI structure of CD59. The mass

Figure 10 Mass analysis of the T9-GPI anchor

T9-GPI peptide was treated with (A) 48% hydrofluoric acid or glycosidases (Table 1b), (B) digest 4, jack bean α -mannosidase, (C) digest 2, bovine testis β -galactosidase, or (D) digest 1, *A. ureafaciens* sialidase.

value data obtained in the present study are consistent with the consensus structure but do not prove it. However, when combined with the biochemical data available [32] the GPI glycan core structure shown in Figure 11 appears highly likely. The main uncertainty concerns the second phosphoethanolamine, whose location remains to be confirmed.

At present the origin of urinary CD59 is unknown. It could originate from the epithelial cells of kidney or from plasma. Using indirect immunofluorescence CD59 has been detected in the epithelial cells of distal tubules, collecting ducts and glomeruli of kidneys [17,46]. Both CD59 and DAF may become shed from epithelial cells. Shedding could occur in the form of small membrane vesicles, with the phospholipid tail left intact, or after cleavage of the anchor by the phospholipase C or phospholipase D enzymes. Recent studies have verified that CD59 is present in small membrane microparticles in the urines of normal individuals as well as of patients with membranous glomerulonephritis [24]. As indicated above, after CD59 has become shed from cell surfaces it may lose the phospholipid portion of the anchor and transform into a soluble hydrophilic form. Alternatively, for a portion of the synthesized CD59 molecules the addition of the lipid tail may have failed and resulted in the secretion of non-lipid-tailed forms. Considering the fact that CD59 is present on all blood cells and on vascular endothelial cells only a little CD59 is present in normal human plasma [23]. As a small and hydrophilic molecule soluble CD59 could become filtered through kidney glomeruli and concentrate into urine, whereas the lipid-tailed forms would remain in the vasculature and

reincorporate into cell membranes or plasma lipoprotein particles [23,24]. The heterogeneity of the oligosaccharide and GPI-anchor structures suggests that soluble urinary CD59 may originate from multiple sources.

The haemolysis-inhibiting activity of soluble CD59_u was notably weaker than that of the membrane form (Figure 1b). The lower activity of the soluble form is consistent with its weaker binding to the cell membrane (Figure 1a), and suggests that quantification of the difference is dependent on conditions used in the assay. Despite showing low haemolysis-inhibiting activity soluble CD59 bound to the TCCs in equivalent quantities to erythrocyte CD59 (Table 2) [11,39]. In recent studies we have demonstrated that CD59_u is also capable of binding to a nascent form of membrane-associated MAC [39]. These results indicate that the specific protein-protein interaction of soluble CD59 with the TCCs does not require the glycolipid anchor. The lipid tail, however, is important for the full cell lysis inhibitory activity, apparently because it facilitates the incorporation of CD59 into the outer leaflet of the lipid bilayer and the intrinsic inhibition of MAC on the cell membrane.

Comparison of the amino-acid sequences of human [7], primate [47] and rat [48] CD59 with mouse Ly-6 antigens [29], snake venom neurotoxins [27], a putative CD59 homologue in Herpes virus saimiri [49] and uPAR [30] suggests that a common folding pattern based on conserved cysteine residues exists within this family of proteins [7,10,49–52]. This proposal has been substantiated by the recent NMR spectroscopic determination of the three-dimensional structure of the polypeptide backbone of

CD59-T9 GPI-anchor structures

Figure 11 Putative GPI-anchor structures of CD59₀ deduced from exoglycosidase treatment of the C-terminal peptide T9-GPI and MALDI-MS

Aliquots of the T9-GPI peptide were treated with various exoglycosidases as shown in Table 1(b) and subjected to MALDI-MS. The $[M+H]^+$ values indicate mass peaks observed after each treatment. Treatment of all peptides with 48% hydrofluoric acid [31] generated an M_r peak of 1440 corresponding to the T9 peptide with an attached ethanolamine (Figure 10a). Structure of the GPI-core oligosaccharide and location of the second ethanolamine-phosphate group are based on analogy with previously published reports [31,42,43,45]. The suggested variant structures were deduced from analysis of mass shifts after treatment with the linkage-specific exoglycosidases. The percentages indicate the approximate proportions of each structure (A–F) present in the original T9-GPI peptide (Figure 6B). Structures C and C' were both present but their relative quantitative ratio could not be determined by MALDI-MS.

human CD59 [25,26] which resembles that of neurotoxins determined earlier by X-ray crystallography [27]. Although the actual positions of the side chains relative to the protein backbone

are not known the N-linked oligosaccharide is attached to the external N-terminal portion of CD59 where it may have an important role in stabilizing the overall structure of the molecule. At the other end, the attachment of CD59 to cell membranes via a GPI anchor is likely to provide the molecule with flexibility that is needed in its interaction with the TCCs.

This study was supported by the Academy of Finland, the Sigrid Juselius Foundation and the University of Helsinki.

REFERENCES

- Müller-Eberhard, H. J. (1986) *Annu. Rev. Immunol.* **4**, 503–528
- Podack, E. R. and Müller-Eberhard, H. J. (1979) *J. Biol. Chem.* **254**, 9808–9814
- Podack, E. R., Preissner, K. T. and Müller-Eberhard, H. J. (1984) *Acta Pathol. Microbiol. Scand. Sect. C* **92** (Suppl. 284), 89–96
- Jenne, D. E. and Tschopp, J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7123–7127
- Choi, N., Nakano, Y., Tobe, T., Mazda, T. and Tomita, M. (1990) *Int. Immunol.* **2**, 413–417
- Sugita, Y., Nakano, Y. and Tomita, M. (1988) *J. Biochem. (Tokyo)* **104**, 633–637
- Davies, A., Simmons, D. L., Hale, G., Harrison, R. A., Tighe, H., Lachmann, P. J. and Waldmann, H. (1989) *J. Exp. Med.* **170**, 637–654
- Holguin, M. H., Fredrick, L. R., Bernshaw, N. J., Wilcox, L. A. and Parker, C. J. (1989) *J. Clin. Invest.* **84**, 7–17
- Okada, N., Harada, R., Fujita, T. and Okada, H. (1989) *Int. Immunol.* **1**, 205–208
- Meri, S. (1994) *Immunologist* **2**, 149–155
- Meri, S., Morgan, B. P., Davies, A., Daniels, R. H., Olavesen, M. G., Waldmann, H. and Lachmann, P. J. (1990) *Immunology* **71**, 1–9
- Rollins, S. A. and Sims, P. J. (1990) *J. Immunol.* **144**, 3478–3483
- Zalman, L. S., Wood, L. M. and Müller-Eberhard, H. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6975–6980
- Schönermark, S., Rauterberg, E. W., Shin, M. L., Loke, S., Roelcke, D. and Hänsch, G. M. (1986) *J. Immunol.* **136**, 1772–1779
- Watts, M. J., Dankert, J. R. and Morgan, B. P. (1990) *Biochem. J.* **265**, 471–477
- Zalman, L. S., Brothers, M. S. and Müller-Eberhard, H. J. (1989) *J. Immunol.* **143**, 1943–1947
- Meri, S., Waldmann, H. and Lachmann, P. J. (1991) *Lab. Invest.* **65**, 532–537
- Rooney, I. A. and Morgan, B. P. (1992) *Immunology* **76**, 541–547
- Rooney, I. A., Atkinson, J. P., Krul, E. S., Schonfeld, G., Polakowski, K., Saffitz, J. E. and Morgan, B. P. (1993) *J. Exp. Med.* **177**, 1409–1420
- Björge, L., Jensen, T. S., Vedeler, C. A., Ulvestad, E., Kristoffersen, E. K. and Matre, R. (1993) *Immunol. Lett.* **36**, 233
- Vedeler, C., Ulvestad, E., Björge, L., Conti, G., Williams, K., Mørk, S. and Matre, R. (1994) *Immunology* **82**, 542–547
- Hakulinen, J. and Meri, S. (1995) *Immunology* **85**, 495–501
- Meri, S., Väkevää, A., Laari, T. and Lachmann, P. J. (1991) *Compl. Inflamm.* **8**, 193 (abstr.)
- Väkevää, A., Jauhainen, M., Ehnholm, C. E., Lehto, T. and Meri, S. (1994) *Immunology* **82**, 28–33
- Lehto, T., Honkanen, E., Teppo, A.-M. and Meri, S. (1995) *Kidney Int.* **47**, 1403–1411
- Fletcher, C. M., Harrison, R. A., Lachmann, P. J. and Neuhaus, D. (1994) *Structure* **2**, 185–199
- Kieffer, B., Driscoll, P. C., Campbell, I. D., Willis, A. C., van der Merwe, P. A. and Davis, S. J. (1994) *Biochemistry* **33**, 4471–4482
- Endo, T. and Tamiya, N. (1987) *Pharmacol. Ther.* **34**, 403–451
- Sugita, Y., Nakano, Y., Oda, E., Noda, K., Tobe, T., Miura, N.-H. and Tomita, M. (1993) *J. Biochem. (Tokyo)* **114**, 473–477
- Shevach, E. M. and Korty, P. E. (1989) *Immunol. Today* **10**, 195–205
- Ploug, M. and Ellis, V. (1994) *FEBS Lett.* **349**, 163–168
- Homans, S. W., Ferguson, M. A., Dwek, R. A., Rademacher, T. W., Anand, R. and Williams, A. F. (1988) *Nature (London)* **333**, 269–272
- Nakano, Y., Noda, K., Endo, T., Kobata, A. and Tomita, M. (1994) *Arch. Biochem. Biophys.* **311**, 117–126
- Ninomiya, H., Stewart, B. H., Rollins, S. A., Zhao, J., Bothwell, A. L. and Sims, P. J. (1992) *J. Biol. Chem.* **267**, 8404–8410
- Menu, E., Tsai, B. C., Bothwell, A. L. M., Sims, P. J. and Bierer, B. E. (1994) *J. Immunol.* **153**, 2444–2456
- Stefanova, I. and Horejsi, V. (1991) *J. Immunol.* **147**, 1587–1592
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

-
- 37 Baumann, M. (1990) *Anal. Biochem.* **190**, 198–208
- 38 Sutton, C. W., O'Neill, J. A. and Cottrell, J. S. (1994) *Anal. Biochem.* **218**, 34–46
- 39 Lehto, T. and Meri, S. (1993) *J. Immunol.* **151**, 4941–4949
- 40 Davitz, M. A., Hereld, D., Shak, S., Krakow, J., Englund, P. T. and Nussenzweig, V. (1987) *Science* **238**, 81–84
- 41 Stahl, N., Baldwin, M. A., Hecker, R., Pan, K.-M., Burlingame, A. L. and Prusiner, S. B. (1992) *Biochemistry* **31**, 5043–5053
- 42 Roberts, W. L., Santikarn, S., Reinhold, V. N. and Rosenberry, T. L. (1988) *J. Biol. Chem.* **263**, 18776–18784
- 43 Redman, C. A., Thomas-Oates, J. E., Ogata, S., Ikehara, Y. and Ferguson, M. A. J. (1994) *Biochem. J.* **302**, 861–865
- 44 Ferguson, M. A., Homans, S. W., Dwek, R. A. and Rademacher, T. W. (1988) *Science* **239**, 753–759
- 45 McConville, M. J. and Ferguson, M. A. J. (1993) *Biochem. J.* **294**, 305–324
- 46 Rooney, I. A., Davies, A., Williams, J. D., Griffiths, D., Meri, S., Lachmann, P. J. and Morgan, B. P. (1991) *Clin. Exp. Immunol.* **83**, 251–256
- 47 Fodor, W. L., Rollins, S. A., Biancocarón, S., Burton, W. V., Guilmette, E. R., Rother, R. P., Zavoico, G. B. and Squinto, S. P. (1995) *Immunogenetics* **41**, 51
- 48 Rushmere, N. K., Harrison, R. A., Vandenberg, C. W. and Morgan, B. P. (1994) *Biochem. J.* **304**, 595–601
- 49 Albrecht, J.-C. and Fleckenstein, B. (1992) *J. Virol.* **66**, 3937–3940
- 50 Leclair, K., Palfree, R. G. E., Flood, P. M., Hammerling, U. and Bothwell, A. (1986) *EMBO J.* **5**, 3227–3234
- 51 Bothwell, A., Pace, P. E. and LeClair, K. P. (1988) *J. Immunol.* **140**, 2815–2820
- 52 Ploug, M., Kjalke, M., Rønne, E., Weidle, U., Høyer-Hansen, G. and Danø, K. (1993) *J. Biol. Chem.* **268**, 17539–17546
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Received 19 January 1996; accepted 22 February 1996