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Structural properties of the glycoplasmanylinositol anchor phospholipid of the complement membrane attack complex inhibitor CD59

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SUMMARY

CD59, the membrane regulator of autologous C5b-9 channel formation, exhibits variable sensitivity to cleavage by phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that releases glyco-inositolphospholipid (GPI)-anchored proteins from cell surfaces. To determine whether the GPI-anchor phospholipid of CD59 is similar to that of decay-accelerating factor (DAF) and whether variation in its structure underlies its variable enzyme susceptibility, the GPI anchors of the two proteins expressed on erythrocytes, polymorphonuclear and mononuclear leucocytes were compared *in situ* and after purification. Flow cytometric analyses of PI-PLC-treated cells showed parallel cell type specific release of both proteins as a function of enzyme concentration. Non-denaturing PAGE analyses of alkaline/hydroxylamine-treated proteins (affinity-purified from [^{125}I]-surface-labelled cells) provided evidence for (i) comparable proportions of GPI-anchor acylation, and (ii) alkali-resistant rather than alkali-sensitive lipid substituents in erythrocytes. These findings argue that the differential C5b-9 sensitivity that distinguishes paroxysmal nocturnal haemoglobinuria II and III erythrocytes does not derive from expression of CD59 molecules with alternative GPI-anchor phospholipid structures.

Keywords complement decay-accelerating factor CD59 GPI anchors paroxysmal nocturnal haemoglobinuria

INTRODUCTION

Recent research [1–9] has identified an ~18–20-kD complement regulatory protein on human blood cell surfaces that restricts plasma membrane insertion and polymerization of autologous complement component C9. Similar to the decay-accelerating factor (DAF), which circumvents deposition of autologous C3b [10–13], this protein, termed CD59 [14,15], is cleavable from cell surfaces *in situ* by incubation with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that removes myo-inositol monophosphate from inositol phospholipids contained in glyco-inositolphospholipid (GPI)-anchor structures [reviewed in 16,17]. Also similar to DAF [13], the protein when purified is able to re-incorporate *in vitro* into cell membranes [1,4,9,18,19]. Additionally, in paroxysmal nocturnal haemoglobinuria (PNH) [20], a disorder traced to abnormal GPI-anchoring [21–23], CD59, like DAF, is deficient in C5b-9 sensitive erythrocytes [4,5,9,18,24].

Because each of the above findings regarding DAF is attributable to its GPI-anchor, the similarities with CD59 have suggested that it is anchored by an equivalent structure. In different studies, however, it exhibited variable sensitivity to PI-PLC-mediated cleavage, whether studied *in situ* [1,2,4,6,9] or purified [25]. Additionally, in PNH a subset of patients exists whose erythrocytes (type II) [26,27] exhibit only partially enhanced (~5- as opposed to ~25-fold greater than normal) sensitivity to autologous complement-mediated injury and lack DAF [13,28], but not the activity attributed to CD59 [13]. These observations have prompted questions of whether CD59's membrane linkage might differ from that of DAF.

Previous characterizations of DAF's GPI-anchor have shown that it exhibits differential inositol acylation [29] and that this structural variation is regulated in a cell-specific fashion [29,30]. Moreover, in erythrocytes it is based on 1-alkyl,2-acyl-glycerol [29]. These structural features contrast with unacylated, dimyristylphosphatidylinositol, which is uniformly present in trypanosome membrane form variant surface glycoproteins (mfVSGs) [16,17], the first protein in which GPI-anchors were chemically characterized. The differences in DAF's anchor

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appear to be characteristic of human GPI-anchors in general, however, because they are also observed in human erythrocyte acetylcholinesterase (AChE) [31,32] and placental alkaline phosphatase (PLAP) [33].

In the present study, the membrane-anchoring mechanism of CD59 was studied both *in situ* and using purified protein. Its structural properties were compared in erythrocytes, polymorphonuclear leucocytes (PMN), and peripheral blood mononuclear cells (PBMC) and the results correlated with those for DAF.

MATERIALS AND METHODS

Proteins, columns, and cells

Murine anti-DAF (MoAb) IA10 [28], murine anti-CD59 MoAb 1F5 [34], and rat anti-CD59 MoAb YTH53.1 [2,35] were prepared as described. Non-relevant murine IgG RPC5 was purchased from Litton Bionetics (Kensington, MD) and rat IgG from Accurate Chemical & Scientific Co (Westbury, NY). *Bacillus thuringiensis* PI-PLC was bought from ICN Biomedicals (Costa Mesa, CA).

Protein A-agarose was obtained from Zymed Labs (San Francisco, CA). IA10-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was prepared as described [36] and YTH53.1- and bovine serum albumin (BSA)-Sepharose were made in a parallel fashion, using 0·8 and 2·9 mg protein per millilitre of swollen gel, respectively.

Erythrocytes and PBMC were separated from EDTA-anticoagulated blood on Ficoll–Paque and PMN from heparinized blood on a discontinuous Percoll gradient composed of 3 ml each of 54%, 63% and 72% Percoll [37].

Flow cytometric analyses

Cells were stained primarily using pre-titred saturating concentrations (2·5 μg/ml) of murine (IA10) anti-DAF [22,28], (IF5) anti-CD59, or control IgG. Murine IgG stained cells were stained secondarily with FITC-F(ab′)$_2$ sheep anti-mouse IgG (Organon Teknika-Cappel, Durham, NC). Fluorescence was quantified on a Cytofluorograf 2S (Ortho Diagnostic Systems, Westwood, MA).

Surface labelling and immunoprecipitations

Cells were labelled with 5 mCi ^{125}I in 5 ml 15 × 85 mm tubes precoated with 300 μg Iodogen [38]. Washed [^{125}I]-labelled erythrocytes and PBMC were extracted on ice as described [29], using 1 ml of phosphate-buffered saline (PBS) containing 1% Triton X-100 (TX-100) (Packard Instruments, Downers Grove, IL), 0·5% deoxycholate (DOC) (Sigma Chemical Co, St Louis, MO), 0·1% SDS, and 1/33 volume of protease inhibitors (1 mg/ml each of antipain, elastinal, leupeptin, chymostatin (Sigma), phosphoramidon, pepstatin A (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 10 μg/ml aprotinin (Sigma)). In the case of PMN, to further control for proteolysis, washed [^{125}I]-labelled cells were extracted by sonication for 15–30 sec in 0·5 ml 2% SDS [31], 0·02 M Tris-HCl, pH 7·4, 0·05 M benzamidine, 0·01 M EDTA, 10 μg/ml aprotinin, 0·001 M phenylmethylsulfonyl fluoride (PMSF), 0·001 M diisopropylfluorophosphate (DFP), and 1/33 volume of protease inhibitors, and the extract diluted with 0·05 M Tris-HCl, pH 7·4, containing 0·19 M NaCl, 0·006 M EDTA, 2·5% TX-100, 0·02% sodium azide, and 10 μg/ml aprotinin to which 0·001 M PMSF, 0·001 M DFP, and 1/33 volume of protease inhibitors had been added. After centrifugation to remove insolubles, extract supernatants were stored at −70°C.

Aliquots of the thawed extracts (pre-absorbed with Protein A agarose) were rotated at 20°C for 30–60 min with 50 μl of packed IA10-, YTH53.1- or BSA-Sepharose, and extensively washed as described [39]. DAF protein was eluted with 0·5 ml of 0·14 M NaCl, 0·05 M diethylamine, pH 11·5, containing either 0·2% Nonidet P-40 (NP-40) (Pharmacia) or TX-100 [36], and CD59 protein was eluted with 0·5 ml 0·1 M sodium acetate, pH 3, with the same detergent [2]. Supernatants containing eluted proteins were dialysed at 4°C against 4 *l* of 0·01 M Tris-HCl, pH 7·4, containing the respective detergent.

Structural analyses

Concentrated dialysates were incubated at 4°C for 18 h with hydroxylamine or buffer, and, after dialysis and re-concentration, aliquots of each were incubated at 37°C for 2 h with PBS alone or containing PI-PLC at a final concentration of 90 or 180 mU/ml as described [29]. SDS–PAGE analyses were performed on slab gels under reducing conditions as described [40]. Audioradiographs were prepared at −70°C on X-OMAT XAR-5 film (Eastman Kodak, Rochester, NY).

Non-denaturing PAGE analyses were performed [29,41,42] using 7·5% gels containing 0·5% TX-100 (or NP-40) and subjected for 3·5 h to 10 V/cm on a water-cooled, horizontal Multiphor II Electrophoresis Unit (Pharmacia) [29]. Autoradiograms of the fixed, dried gels were prepared, then analysed by densitometry on an LKB 2400 Ultroscan SL Laser Densitometer (Pharmacia) interfaced with the GelScan XL version 1.20 utility, run on a Tandon Targa computer.

In densitometric analyses, the sum of the area under the signals for detergent-associated and detergent-unassociated radioactivity was defined as 100%. The percentage of unsubstituted inositol was taken from the percentage of detergent-unassociated radioactivity in lane 2. The percentage of substituted inositol was derived by subtracting the percentage of detergent-associated radioactivity in lane 2 from that in lane 4. The percentage of base-resistant-phospholipid was taken as the percentage of detergent-associated radioactivity in lane 3.

In some experiments, some radioactivity remained in the wells and failed to enter the non-denaturing PAGE gels, presumably as a consequence of concentrating samples before loading. In computations, signals corresponding to these bands were excluded based on the assumption that different GPI-anchor structures were not selectively retained.

RESULTS

Cell-dependent differences in susceptibility of CD59 to PI-PLC cleavage

To determine if the efficiency of PI-PLC-mediated release of CD59 from cell surfaces varies in different blood cell types similarly to that of DAF, erythrocytes, PMN and PBMC were incubated at 37°C for 1 h with graded concentrations of the enzyme or with buffer control. Aliquots of the treated cells stained with IA10 anti-DAF or 1F5 murine anti-CD59 MoAbs (to allow use of the same FITC-labelled anti-immunoglobulin) were analysed by flow cytometry. As shown in Fig. 1a (assuming that IA10 and 1F5 bind singly with high affinity to their respective antigens and that their binding is not differentially influenced by steric factors) the relative expression levels of

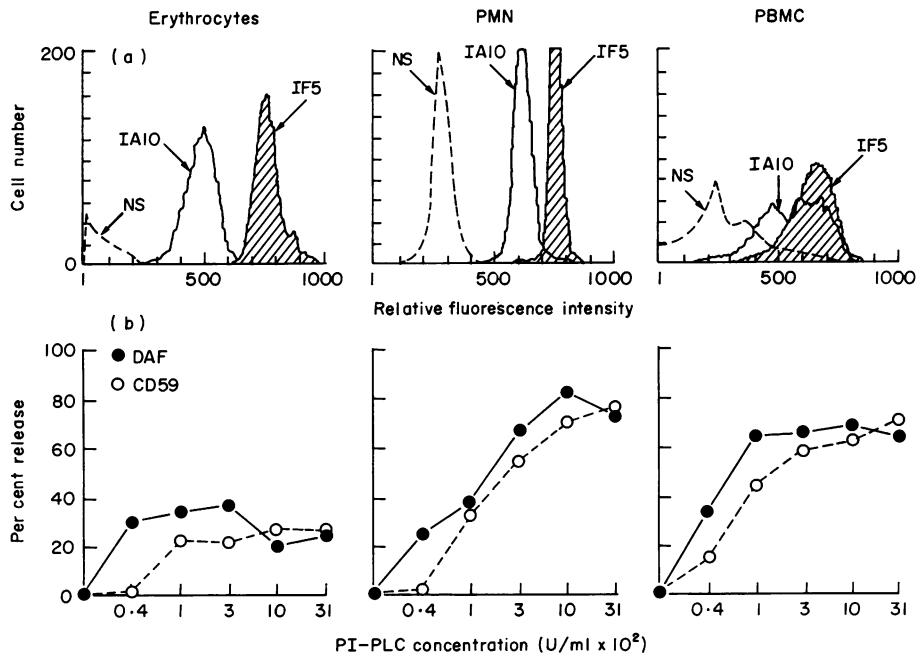

Fig. 1. (a) Erythrocytes, polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) were stained with anti-DAF (IA10), anti-CD59 (1F5), or irrelevant RPC5 MoAb and FITC sheep anti-mouse IgG secondarily-stained cells analysed by flow cytometry at constant fluorescent gain (so as to allow comparisons between DAF and CD59 surface densities). (b) The three cell types were incubated at 37°C for 1 h with buffer alone or with increasing concentrations of PI-PLC and the treated cells analysed as above. Per cent release of delay-accelerating factor (DAF) and CD59 protein from the cells is shown as a function of the initial (that is, ×2 concentrated) PI-PLC concentration. NS, Not significant.

CD59 in erythrocytes PMN and PBMC appear higher than those of DAF. Moreover, expression of CD59 in PBMC is homogeneous in contrast to that of DAF which is heterogeneous among lymphocyte subpopulations and overlaps with the negative control [28, 43–46]. In Fig. 1b it can be seen that, whereas no more than ~20–25% of CD59 was removable from erythrocytes regardless of enzyme concentration, ~70–85% was removable from PMN and PBMC. This pattern of release conformed closely to that of DAF.

Comparative analyses of CD59's GPI-anchor structure in different blood cell types

To establish whether the cell type variations in PI-PLC release of CD59 *in situ* reside in CD59's GPI-anchor structure rather than other cell factors, isolated erythrocytes, PMN and PBMC were [^{125}I]-surface-labelled, and the labelled CD59 and DAF proteins were affinity purified from cell extracts. SDS–PAGE analyses established that the isolated products, shown in Fig. 2, were free of contaminants and structurally intact, appropriate for comparative analyses of their anchor structures. The diffuse CD59 bands on SDS–PAGE reflect CD59's extensive glycosylation [6,47]. Portions of each protein were treated alternatively with buffer, PI-PLC, hydroxylamine, or hydroxylamine and PI-PLC as described in Materials and Methods [29]. SDS–PAGE analyses (under reducing conditions) (Fig. 2) showed that, although PI-PLC treatment of CD59 induced a slight decrease in mobility, the chemical and enzymatic exposures did not otherwise alter CD59 protein structure. The decrease in mobility noted for CD59 contrasted with an increase in that of DAF [36,48].

The results of non-denaturing PAGE analyses of the variously treated CD59 proteins are shown in Fig. 3, with densitometric comparisons to DAF given in Table 1. In the case of erythrocytes, >95% of the CD59 protein treated with PI-PLC alone exhibited slow migration (similar to that in the buffer-treated control) indicative of detergent micellar association. Less than 5% exhibited more rapid migration, characteristic of a detergent-free hydrophilic species. This compared with <5–15% of DAF (Table 1). In contrast, 88–90% of the protein pre-treated with hydroxylamine (which cleaves ester bonds) and then PI-PLC, was released as a hydrophilic species. This again paralleled DAF (76–90% release).

Differently from findings with erythrocytes, PI-PLC alone cleaved large percentages of CD59 and DAF proteins derived from the surfaces of leukocytes. In the cases of PMN and PBMC, 60–95% and 71–86% of PI-PLC-treated CD59 protein migrated as hydrophilic species. This compared with 75–90% and 71–83% of DAF (Table 1). In both of these cell types, up to 95% of the hydroxylamine- and then PI-PLC-treated CD59 protein, respectively, ran as hydrophilic species indicating that the residual PI-PLC-resistant molecules in both cases were rendered enzyme sensitive by the hydroxylamine deacylation. These results conform to those of DAF (Table 1), in which up to 95% was released from PMN and PBMC [29].

Only 5–18% of erythrocyte-associated CD59 was released from detergent following treatment with hydroxylamine alone, consistent with an alkylacylglycerol-based GPI-structure, whereas somewhat more was released from PMN and PBMC. Similar results were found for DAF [29].

DISCUSSION

The experiments in this study provide evidence that the membrane-anchoring mechanism of CD59 (i) exhibits dose-

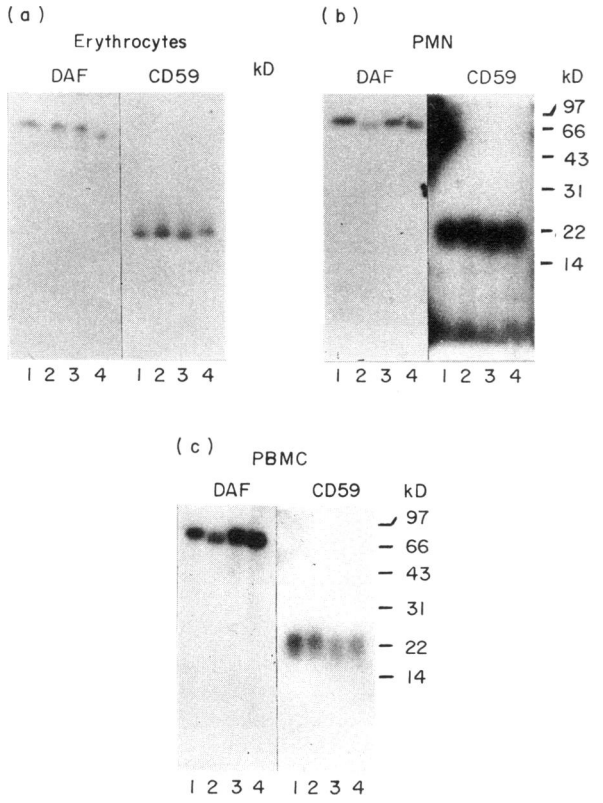

Fig. 2. [^{125}I]-labelled CD59 and delay-accelerating factor (DAF) proteins, affinity-purified from extracts of surface-labelled erythrocytes (a), polymorphonuclear leucocytes (PMN) (b), and peripheral blood mononuclear cells (PBMC) (c) were incubated with hydroxylamine or buffer control. Dialysed and concentrated samples were incubated at 37°C for 2 h with buffer or 1/50 to 1/100 PI-PLC and analysed on 12·5% SDS–PAGE gels. Lane 1, buffer alone; lane 2, buffer then PI-PLC; lane 3, hydroxylamine then buffer; lane 4, hydroxylamine then PI-PLC. For each cell type, DAF and CD59 protein preparations were loaded onto the same gel, but the autoradiographs were exposed for different times.

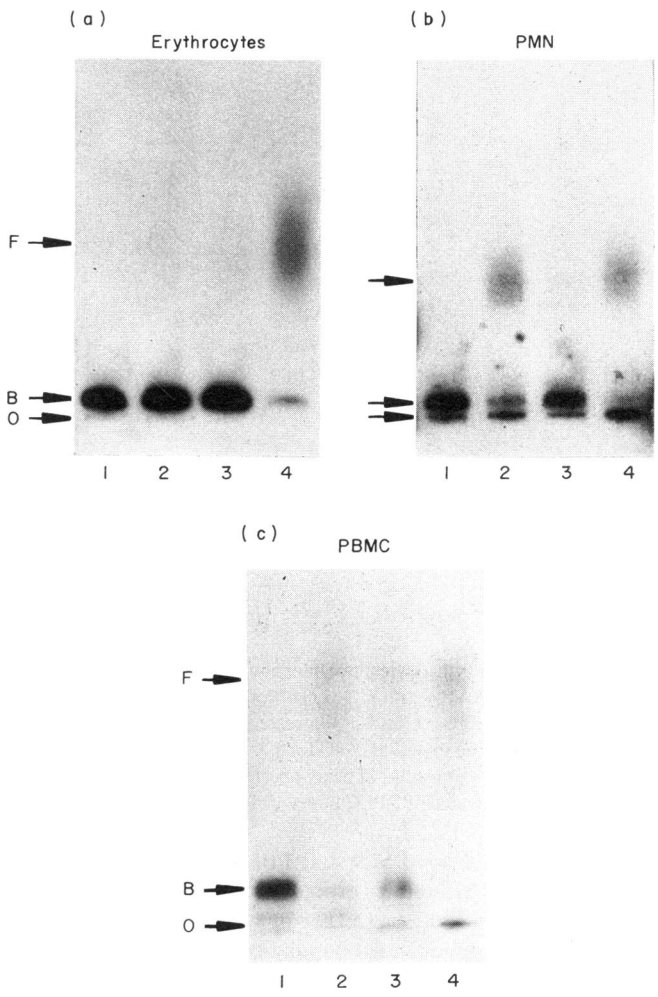

Fig. 3. The variously treated CD59 samples from erythrocytes, polymorphonuclear leucocytes (PMN), and peripheral blood mononuclear cells (PBMC) described in the legend for Fig. 2 were subjected for 3·5 to 7·5% non-denaturing PAGE in the presence of 0·5% NP-40 or TX-100. The resulting autoradiographs are shown in (a), (b) and (c). Lane designations are the same as in Fig. 2. O, Origin; B, detergent-bound; F, free. In (c), the faint band visible between the origin (O) and the major bound moiety (B) (lanes 1 and 2) has been excluded from the densitometric analysis in Table 1. Delay-accelerating factor (DAF) and CD59 protein preparations were loaded as in Fig. 2.

dependent enzyme and mild alkaline cleavage susceptibility characteristic of a GPI moiety, (ii) shows cell-specific variations in inositol acylation, and (iii) contains a phospholipid which resists alkaline hydroxylamine cleavage. These structural features parallel those of DAF and other GPI-anchored proteins that have been analysed to date in human cells, i.e. erythrocyte AChE [31,32] and PLAP [33].

CD59 was identified as a GPI-anchored membrane complement regulatory protein independently by several laboratories. Using antibodies raised after purification of its functional activity, Sugita *et al.* [1,47] reported that only small amounts of the factor (which they termed membrane attack complex inhibitory factor) were detectable in the supernatant of erythrocytes incubated with PI-PLC. With antisera prepared following a different functional purification protocol, Holguin *et al.* [4,25] found that 10% of the protein (which they termed membrane inhibitor of reactive lysis (MIRL)) was released from erythrocytes by PI-PLC, whereas 44% was released from K562 erythroleukaemic cells. Stefanova *et al.* [6] purified the protein independently of its functional activity by affinity chromatography, exploiting a murine MoAb initially designated MEM-43 and subsequently named CD59 [14,15]. In flow cytometric assays of MEM-43-stained human PBMC, PI-PLC treatment caused a substantial decrease of cell-associated antigen. On Western blots, no residual antigen was detectable in extracts of enzyme-treated HPB-ALL cells. Whitlow *et al.* [9] used a similar approach relying on another murine MoAb, H19 [3]. In flow cytometric assays of H19-stained PBMC, PI-PLC treatment released 80% of the protein. Using a rat anti-lymphocyte MoAb, YTH53.1 [35], Davies *et al.* [2] identified the mRNA encoding the protein by flow cytometric assays of COS cells transfected with a cDNA library. Almost all of the expressed protein (which they equated to CD59 antigen) was PI-PLC sensitive. N. Okada *et al.* [5,18,34] isolated the protein by affinity chromatography with yet another murine MoAb, 1F5. In flow cytometric assays of 1F5-stained human T cell lymphotropic virus ((HTLV)-I infected) MT2 cells and nylon wool purified T cells, PI-PLC completely removed the protein (which they called HRF20) [49]. It was subsequently reported [45] that

Table 1. ND-PAGE comparisons of CD59 and DAF GPI anchor structures

		Unsubstituted inositol, lane 2, % free		Substituted inositol, lane 4, % free minus lane 2, % free		Alkali-resistant phospholipid lane 3, % bound	
Cell type	Donor	CD59	DAF	CD59	DAF	CD59	DAF
Erythrocytes	A1	<5	11	88	66	>95	88
	A2	<5	<5	90	78	>95	>95
	A3	5	15	84	75	82	86
PMN	A	60	75	9	18	>95	>95
	B	95	90	3	11	66	82
PBMC	C	86	83	14	7	55	68
	D	71	71	29	29	55	76

PMN, Polymorphonuclear leucocytes; PBMC, peripheral blood mononuclear cells.

80%, 54%, and 55% of the 1F5 antigen was PI-PLC releasable from blood lymphocytes, monocytes, and PMN while the parallel release of DAF from these cell types was 75, 48, and 32%, respectively.

Our observation that CD59's GPI-anchor exhibits non-denaturing PAGE properties of cell-specific inositol acylation accounts for the previously reported variability in CD59's PI-PLC sensitivity. Our finding that 84–90% of CD59 molecules in erythrocytes contain GPI-anchors that exhibit acylation can additionally explain observations by Holguin *et al.* [25] that PI-PLC treatment of purified erythrocyte MIRL did not prevent its incorporation into PNH erythrocytes or alter its ability to inhibit C9 polymerization in the reconstituted PNH cells. The reason in their studies for the apparent discrepancy between the PI-PLC-sensitivities of *in situ* and purified CD59 protein is unclear, but selective loss of unacylated molecules during purification could lead to a higher proportion of molecules with acylated GPI-anchors in the latter case. Our observation that PI-PLC-cleaved CD59 molecules exhibit decreased SDS–PAGE mobility can also account for previous observations [25] that (i) MIRL released from erythrocytes by the enzyme displayed a slightly greater apparent *M*r than MIRL immunoprecipitated from intact cells, whereas (ii) when isolated, PI-PLC-treated MIRL showed the same apparent *M*r as untreated MIRL. Previous findings that GPI-anchor removal from VSGs, Thy-1 antigen, and AChE is not associated with a change in SDS–PAGE mobility [16], while that from erythrocyte DAF is associated with an increase [36,48] and that from LFA-3 with a decrease [50], indicate that charge and/or conformational factors may operate in this context in a protein-specific manner. The apparent *M*r variation due to these factors should be distinguished from the finding that PMN-derived DAF [28], displays greater apparent *M*r than erythrocyte associated DAF protein, an *M*r difference in this case arising from variations in post-translational glycosylation.

The failure of mild alkali treatment to release erythrocyte-derived CD59 from detergent argues that the inositol phospholipid in CD59's GPI-anchor, like those in erythrocyte-derived AChE's and DAF's GPI-anchors [29,31,32], is based on alkylacyl- rather than diacyl-glycerol as present in mfVSGs of *Trypanosoma brucei* [16,17]. Gas chromatographic analyses of the GPI-anchors in the latter two human proteins have shown that their alkyl side chains consist principally of C18:0 and C18:1 alkylglycerols and that their acyl substituents include both saturated, e.g. C16:0 and C18:0, and unsaturated hydrocarbons, e.g. C22:4 and C22:5. The composition of CD59's GPI-anchor remains to be determined but the close parallel that has been noted between the anchor compositions of DAF and AChE in erythrocytes suggests it will be similar. Whether the greater alkali susceptibility of leucocyte-derived CD59 reflects diacylglycerol will require further study. The reason why some CD59 protein resisted PI-PLC cleavage after pre-incubation with hydroxylamine (lane 4, bound) is unclear. Possible explanations include (i) incomplete enzymatic cleavage; (ii) incomplete deacylation by hydroxylamine; or (iii) an alternatively anchored CD59 form.

The biological relevance of GPI-anchor structural variability among different blood cell types remains incompletely understood. The additional acylation present in erythrocyte associated anchors renders these structures resistant to cleavage by PI-PLC and less sensitive to PI-PLD cleavage when purified [29,51]. This enzyme resistance may enhance the membrane stability of these proteins over time consistent with the longer (120 day) life span in the circulation of erythrocytes as compared with leucocytes.

The molecular basis for the differential resistance of PNH II erythrocytes to C5b-9 mediated injury [27] is unknown. If the GPI-anchor structure of CD59 on PNH II erythrocytes does not differ from that of CD59 (and DAF) on normal erythrocytes, a defect in a GPI-anchor assembly or attachment enzyme should affect expression of both proteins in an equivalent manner. Findings that LFA-3 and FcγRIII exist in both GPI- and conventionally-anchored forms [50,52–57], and that in both cases only the conventionally anchored PI-PLC-resistant species is detectable in affected erythrocytes [58–60] provide precedents for the possibility that an alternative, perhaps conventionally anchored form of CD59 may exist. Alternatively, CD59 expression may be diminished similarly to DAF expression [24] but sufficient function retained at lower levels, or another protein, e.g. homologous restriction factor/C8-binding protein [61–63], with parallel functional activity may be differentially expressed in PNH II cells.

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