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Shedding and enrichment of the glycolipid-anchored complement lysis inhibitor protectin (CD59) into milk fat globules

J. HAKULINEN & S. MERI *Department of Bacteriology and Immunology, University of Helsinki, Helsinki, Finland*

SUMMARY

Protectin (CD59) is a glycolipid-anchored inhibitor of the membrane attack complex (MAC) of human complement (C) that protects blood cells, endothelial cells and various epithelial cells from C-mediated lysis. Because of its activities protectin is a candidate molecule for use in the treatment of paroxysmal nocturnal haemoglobinuria or conditions where MAC causes tissue damage. Soluble, phospholipid-free forms of protectin have been isolated from human urine and produced in recombinant form, but they have only a relatively weak C lysis-inhibiting activity. In the present study we have looked for functionally active protectin in human breast milk. Milk is rich in fat droplets, milk fat globules (MFG), that are enveloped in a plasma membrane derived from secretory cells of the mammary gland. The membranes of MFG contain a variety of glycoproteins expressed by the mammary epithelial cells. Both immunofluorescence and immunoblotting analysis demonstrated that protectin was strongly expressed on human MFG. In sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis, MFG protectin ($CD59_M$) appeared as distinct bands with apparent molecular weights of 19 000–23 000 MW, similar to protectin extracted from MCF7 breast carcinoma cells. $CD59_M$ in breast milk was functionally active and had a glycophospholipid anchor, as judged by its ability to incorporate into guinea-pig erythrocytes and inhibit their lysis by human complement. These results indicate that functionally active protectin becomes enriched in MFG and imply that secretion of glycophospholipid-anchored molecules, e.g. into cow milk and colostrum, could be exploited as a means of producing bioactive molecules that need to be targeted into cell membranes.

INTRODUCTION

Human milk is a complex fluid that provides the essential nutrients for infants during their first months of life. Apart from being a source of energy and anabolic components, milk also has a functional role in an infant's ability to resist infections. The constituents of milk are physically compartmentalized into a water-phase, milk fat globules (MFG) and immunoreactive cells.[1] Human milk contains 4% lipid that is secreted as droplets of 1–10 μm in diameter. The droplets are enveloped in a plasma membrane derived from the mammary secretory cells.[2] The MFG membranes contain a variety of glycoproteins that have been used to raise antibodies able to detect surface antigens even on malignant human mammary cells.[3,4]

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Abbreviations: C, complement; $CD59_M$, breast milk protectin; GPE, guinea-pig erythrocyte; GPI, glycophosphoinositol; MAC, membrane attack complex; MFG, milk fat globules; PNH, paroxysmal nocturnal haemoglobinuria; TCC, terminal complement complexes.

Correspondence: Dr S. Meri, Department of Bacteriology and Immunology, PO Box 21 (Haartmaninkatu 3), FIN-00014 University of Helsinki, Finland.

Protectin (CD59)[5] is an inhibitor of the membrane attack complex (MAC) of complement that is widely distributed on human blood cells[6] and in various tissues.[7] Protectin blocks formation of MAC by preventing C5b–8 catalysed insertion of C9 into lipid bilayers.[8,9] Protectin, as well as two other inhibitors of complement, decay-accelerating factor (DAF; CD55)[10] and C8bp homologous restriction factor (HRF)[11,12] is anchored to cell membranes via a glycophosphoinositol (GPI) moiety that forms a link between the C-terminal amino acid (Asn77 in protectin) of the extracellular polypeptide and the membrane phospholipid.[6,13–15] In addition to various cell membranes, phospholipid-tailed protectin has been found in amniotic fluid and in seminal plasma, where it has been shown to be associated with extracellullar organelles called prostasomes.[16]

Soluble, hydrophilic forms of protectin, which lack the anchor phospholipid,[17] have been detected in various body fluids like urine, tears and saliva[6,18] and produced in recombinant form.[19] Although the soluble form of protectin binds specifically to the terminal C complexes, it has only a weak cytolysis inhibitory activity.[20] In contrast, the phospholipid-tailed form of protectin, by virtue of its ability to become incorporated into cell membranes, is an effective inhibitor of MAC.

Our preliminary studies,[18] as well as those of Bjørge *et al.*,[21] have indicated that protectin is present in human breast milk. This finding is in accordance with the strong expression of protectin in breast duct epithelia as well as in breast tumours and in the breast cancer cell lines (MCF7 and T47D).[22] In the present study we demonstrate that protectin is found in human breast milk in a functionally active GPI phospholipid-tailed form on the surface of MFG.

MATERIALS AND METHODS

Antibodies and sera

The rat hybridoma cell line producing the YTH53.1 anti-protectin monoclonal antibody (mAb) (IgG2b) was kindly provided by Professor H. Waldmann (Department of Pathology, University of Cambridge, UK) and the BRIC-229 (anti-protectin) mouse mAb (IgG2b) was purchased from Bio-Products Laboratory (Elstree, UK). Fluorescein isothiocyanate (FITC)-conjugated antibodies against mouse IgG were purchased from Dakopatts (Copenhagen, Denmark). YTH53.1 IgG was isolated by protein G affinity chromatography (Pharmacia-LKB Biotechnology, Uppsala, Sweden). Normal human serum (NHS) was obtained from healthy laboratory personnel and stored in small aliquots at −70°. Human serum deficient in C9 (C9DS) was prepared from NHS by immunoprecipitating C9 by a polyclonal goat IgG antibody against human C9 (Quidel Corp., La Jolla, CA).

Isolation of human MFG and MFG membranes

Fresh human breast milk samples were obtained from volunteer donors at the Department of Obstetrics and Gynaecology, University of Helsinki, Finland, within 1 week post-partum. The MFG were isolated as described elsewhere.[23] Briefly, milk was centrifuged at 3000 *g* for 15 min at +30°. During centrifugation MFG, because of their low density, float to the top of the gradient and heavier particles like cells sediment to the pellet. The cream layer on the top containing MFG was collected and washed four times with 0·05 M Tris–HCl buffer, pH 7·5, containing 0·25 M sucrose and 1 mM $MgCl_2$. During each wash material in the pellet and aqueous phase was separated from the cream. The washed cream was suspended in phosphate-buffered saline (PBS; 140 mM NaCl, 0·27 mM KCl, 0·15 mM KH_2PO_4, 0·82 mM Na_2HPO_4 and 0·02% NaN_3, pH 7·4) at a ratio of 33% (w/v). The mixture was subjected to two cycles of freezing (−20°) and rapid thawing to +35°. MFG membranes were isolated by centrifugation at 100 000 *g* for 1 hr at +5° and washed twice with PBS.

Immunofluorescence (IF) microscopy

Staining of MFG for indirect IF microscopy was performed as follows. The washed MFG were incubated for 30 min at room temperature with the primary antibody (BRIC-229) diluted in PBS at a concentration of 20 μg/ml, or with PBS only (control). After two washes with PBS (3000 *g* for 10 min at +30°) the FITC-conjugated anti-mouse IgG antibody in PBS was added. After a 30-min incubation, the MFG were washed twice with PBS and fixed with 3% formaldehyde in PBS. As an additional control, MFG particles were treated (30 min at 30°) with phosphatidylinositol-specific phospholipase C (PIPLC) from *Bacillus cereus* (Sigma, St Louis, MO) at 2 IU/ml. The cells in milk were immunostained for CD59 as for MFG, except that the incubations were carried out on ice and cells centrifuged at 500 *g* for 5 min. Staining was controlled by using RPMI-1640 with 10% fetal calf serum (FCS) instead of the primary antibody.

Purification of breast milk protectin ($CD59_M$)

YTH53.1 mAb was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) at a concentration of 2·5 mg IgG/ml gel. Human milk (500 ml) was centrifuged at 800 *g* for 5 min to remove cells. Supernatant was mixed with 0·3% (final concentration) Nonidet P-40 (NP-40) and ultracentrifuged at 100 000 *g* for 1 hr at +5°. During centrifugation the preparation separated into four phases. The solidified fat layer on the top and the pellet were discarded. The aqueous phase was applied on a YTH53.1–Sepharose affinity column stabilized with PBS containing 0·01% NP-40. The unbound material was removed by washing with 0·1% NP-40 in PBS. Protein was eluted with 0·1 M glycine–HCl, pH 2·7, containing 0·01% NP-40. The pH of the collected fractions was set to neutral with 1·0 M Tris–HCl, pH 9·0. Elution of protectin was followed by dot blotting by applying a 1-μl sample from each fraction onto a nitrocellulose membrane that was subsequently immunostained for protectin, as described below. Fractions positive for protectin were pooled and dialysed against PBS with 0·01% NP-40. The protein concentration (210 μg/ml) was determined according to a modified Lowry method[24] and the purity analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).[25] The yield of CD59 in two separate affinity purifications was 1·5 and 2 μg of protein/ml of raw milk. The affinity-purified $CD59_M$ was radiolabelled with sodium-^{125}I to an initial specific activity of $9{\cdot}2 \times 10^6$ c.p.m./μg using the Iodogen method (Pierce Chemical Co., Rockford, IL).

Enzyme treatments

Samples of affinity-purified $CD59_M$ (3·2 μg in a final volume of 40 μl of PBS) were treated with 1 U/ml of PIPLC from *B. cereus* or with 0·1 U/ml of neuraminidase from *Vibrio cholerae* (Behringwerke, Marburg, Germany) for 2 hr at +37°, or with 0·1 U/ml of endoglycosidase F from *Flavobacterium meningosepticum* (Boehringer Mannheim, Mannheim, Germany) for 16 hr at +37°. Prior to endoglycosidase F treatment. the $CD59_M$ preparation was treated with 0·2% SDS and 1% β-mercaptoethanol for 3 min at +100°. Protein samples (0·4 μg of $CD59_M$) after each enzyme treatment were subjected to a 15% SDS–PAGE gel under reducing conditions and stained with silver nitrate.

Immunoblotting of milk protectin

Proteins were electrophoresed on a 15% SDS–PAGE slab gel under non-reducing conditions and transferred to a nitrocellulose filter with a pore size of 0·45 μm (Schleicher & Schuell, Dassel, Germany), according to the method of Towbin *et al.*[26] After blocking non-specific binding sites with 3% bovine serum albumin (BSA) in PBS, the nitrocellulose strips were incubated with the BRIC-229 mAb (11 μg/ml) in 3% BSA/PBS for 1 hr. In the control strips the primary antibody was omitted. Bound antibodies were visualized using alkaline phosphatase-conjugated rabbit antibody against mouse IgG (Zymed, San Francisco, CA) and nitroblue tetrazolium (Sigma) with 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim) as a substrate.

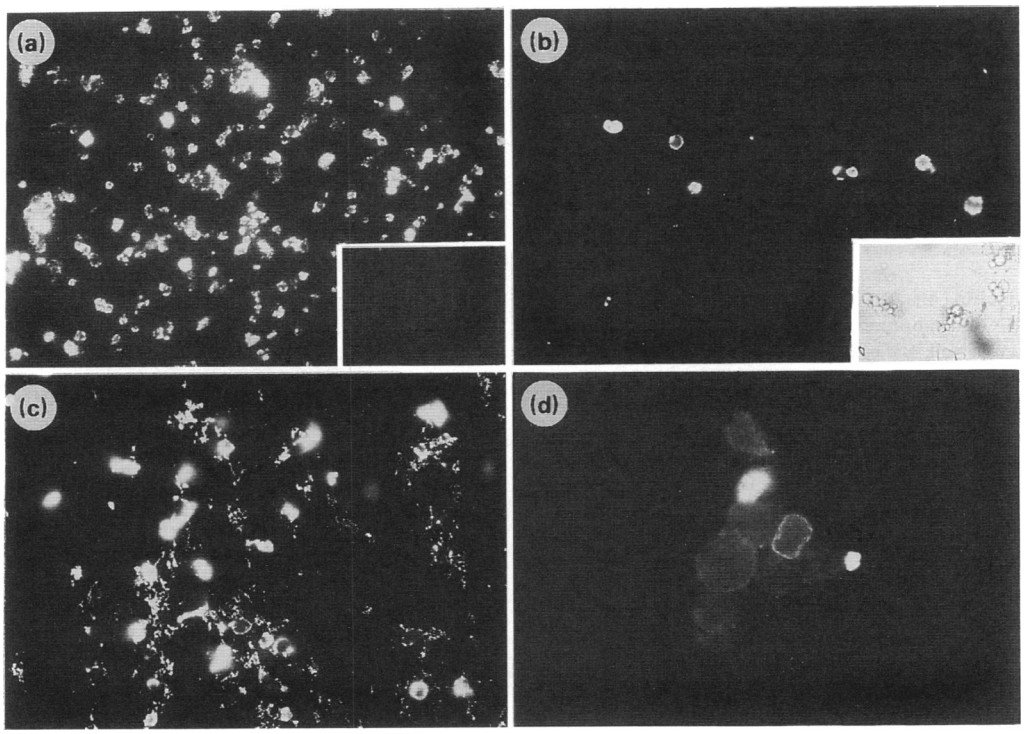

Figure 1. Demonstration of protectin on MFG and on cells isolated from milk by indirect immunofluorescence microscopy. The washed MFG particles (a–c) and cells from human colostrum (d) were incubated with the BRIC-229 anti-CD59 mAb, FITC-conjugated anti-mouse IgG antibody and fixed with 3% formaldehyde. In the control the primary antibody was omitted and MFG particles were treated with the fluorescein-conjugated anti-mouse IgG antibody alone (inset in A). A phase-contrast microscopic image of MFG is shown in the inset of (b). (a) and (b) depict protectin on the surface of MFG particles. Aggregates of lipid-tailed protectin not associated with MFG particles are shown in (c). Protectin on the surface of cells isolated from milk shows a mixed pattern of expression (d). Magnifications (a, c) ×375; (b, d) ×563.

Incorporation of $CD59_M$ into cell membranes and inhibition of complement lysis

The affinity-purified $CD59_M$ was incorporated into guinea-pig erythrocyte (GPE) membranes by incubating (30 min, +37°) radiolabelled $CD59_M$ with 2×10^7 GPE in 50 μl. The amount of $CD59_M$ incorporated into GPE was determined after washing the cells twice with PBS. For a complement lysis test various amounts of $CD59_M$ were incorporated into GPE, which were subsequently incubated with NHS (1/24 dilution) in 60 μl of veronal-buffered saline (VBS; 142 mM NaCl and 5·6 mM Na-5,5-diethylbarbiturate, pH 7·35) for 20 min at +37°. VBS was added to each tube (*ad* 1 ml) and haemolysis determined from adsorbance at 412 nm (OD_{412}) of the supernatants after centrifugation. For total lysis, water was added instead of VBS. Background lysis was obtained from cells treated with VBS instead of NHS.

Binding of $CD59_M$ to terminal complement complexes (TCC)

Twenty microlitres of ^{125}I-labelled $CD59_M$ (90 ng) was incubated with 60 μl of C9DS and 4% inulin for 30 min at +37°. In the control inulin was omitted from the reaction mixture. To separate the unbound and TCC-bound ^{125}I-$CD59_M$, the mixture was layered on the top of a 10–50% sucrose gradient containing 0·01% of NP-40 detergent, and ultracentrifuged for 17 hr at 200 000 *g*. Fractions of 200 μl were collected and counted for radioactivity.

RESULTS

Immunofluorescence demonstration of protectin on MFG particles

MFG were isolated from human colostrum and milk by successive centrifugation. In phase-contrast microscopy, the apparent MFG in the washed cream layer appeared heterogeneous in size and showed no internal structures. When the MFG particles were immunostained with the BRIC-229 mAb (mouse IgG2b), they were found to be covered with the CD59 antigen (Fig. 1A, B). The CD59-specific fluorescence was not homogeneous but appeared in clusters on the surface of the MFG particles. Occasionally CD59-specific staining was seen on aggregates outside the MFG particles (Fig. 1C). When the MFG particles were treated with the fluorescein-conjugated anti-mouse IgG antibody alone, no staining of the MFG was observed. MFG particles subjected to treatment with PIPLC lost their organized structure and immunoreactivity with the BRIC-229 mAb. In line with earlier studies demonstrating protectin on cultured breast tumour cell lines,[22] protectin was found to be expressed on cells in breast milk (Fig. 1D).

Immunoblotting and SDS–PAGE analysis of $CD59_M$

Affinity-purified protectins from milk and MFG membranes were analysed by SDS–PAGE and immunoblotting using the

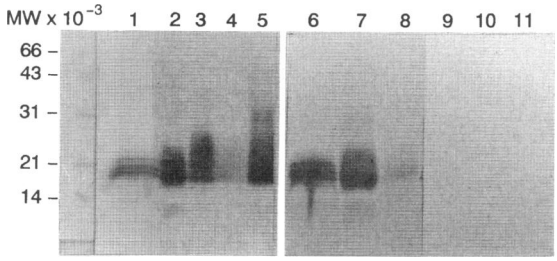

Figure 2. Immunoblotting analysis of protectin in breast milk. Protectin samples from MFG membranes (lanes 1 and 6), affinity-purified protectin from human milk (lanes 2 and 7), MCF7 breast cancer cell line (lane 3), human heart (lane 4), erythrocyte membranes (lane 5) and urine (lane 8) were run on a 15% SDS–PAGE slab gel under non-reducing conditions and transferred to nitrocellulose. Protectin was detected using the BRIC-229 mouse mAb. Bound antibody was immunostained by using alkaline phosphatase-conjugated rabbit antibody against mouse IgG. Lanes 9, 10 and 11 represent controls (protectin from MFG membranes, milk and urine, respectively) where no primary antibody was added. Molecular weight standards are shown on the left.

BRIC-229 mAb. In the MFG membrane preparation the BRIC-229 anti-CD59 mAb bound reproducibly to discrete bands, with apparent molecular weights ranging from 19 000 to 23 000 MW (Fig. 2). The pattern of distinct bands, usually three or four in number, was seen in all MFG samples examined. Control protectin isolated from human erythrocytes ($CD59_E$) was visible as a diffuse smear from 18 000 to *c.* 28 000 MW. $CD59_M$ resembled protectin isolated from MCF7 breast carcinoma cells that also had distinct bands in the immunoblot. The smear for soluble CD59 isolated from human urine ($CD59_U$) started from a slightly higher apparent molecular weight (21 000 MW) than that of the lipid-tailed CD59 from heart and MFG (Fig. 2). In immunoblotting

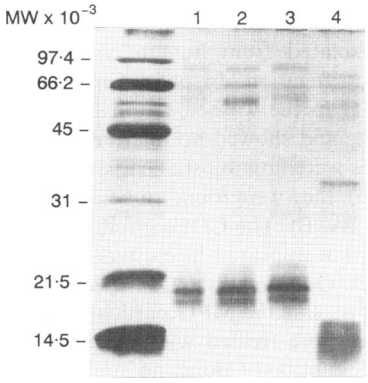

Figure 3. SDS–PAGE analysis of protectin extracted from human milk ($CD59_M$; lane 1) and $CD59_M$ treated with PIPLC (1 U/ml, 2 hr at +37°) from *B. cereus* (lane 2), neuraminidase from *V. cholerae* (0·1 U/ml, 2 hr at +37°) (lane 3) and endoglycosidase F from *F. meningosepticum* (0·1 U/ml, 16 hr at +37°) (lane 4). Prior to endoglycosidase F treatment the $CD59_M$ preparation was treated with 0·2% SDS and 1% β-mercaptoethanol for 3 min at +100°. Protein samples (0·4 μg) of each digestion were run on a 15% SDS–PAGE slab gel under reducing conditions and visualized by silver staining. Molecular weight standards are shown on the left.

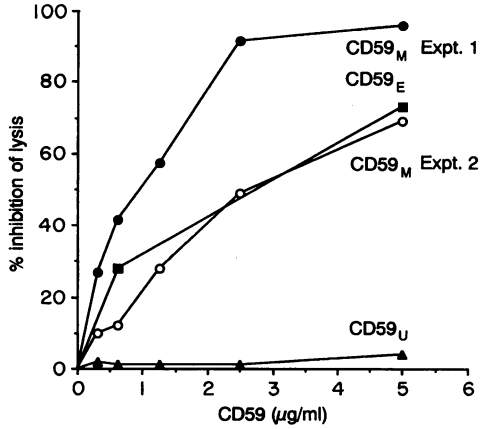

Figure 4. Inhibition of complement lysis of GPE by breast milk protectin. Results shown are from two separate experiments. GPE (2×10^7/ml) were incubated with the indicated amounts of affinity-purified protectin from milk ($CD59_M$), erythrocytes ($CD59_E$) or urine ($CD59_U$) prior to treatment (30 min, +37°), with a 1/24 dilution of NHS in VBS. Lysis was quantified as release of haemoglobin into supernatant (A_{412}). Lysis of GPE was inhibited by the lipid-tailed $CD59_M$ and $CD59_E$ but not by the soluble $CD59_U$.

controls, where the primary antibody was omitted, no reactivity was detected.

In SDS–PAGE the affinity-purified $CD59_M$ migrated as bands with molecular weights of 19 000, 20 000, 22 000 and 23 000 (Fig. 3). When $CD59_M$ was treated with PIPLC or neuraminidase, no apparent change or only a minor shift in mobility in SDS–PAGE was observed. On the other hand, when $CD59_M$ was treated with endoglycosidase F its apparent molecular weight decreased to 14 000–16 000 in a 15% SDS–PAGE slab gel under reducing conditions.

Functional activity of milk protectin

When ^{125}I-labelled $CD59_M$ (14 ng, 12 000 c.p.m.) was incubated with GPE (2×10^7 cells in 50 μl of PBS), 29% of the radioactivity became incorporated into the cells and remained there after repeated cycles of washing. Under similar conditions only 4% of urinary CD59 became associated with the cells. When GPE (2×10^7 cells in 60 μl) were incubated with different amounts of $CD59_M$, their lysis by NHS could be inhibited in a dose-dependent fashion (Fig. 4). Full inhibition of GPE lysis by 4% NHS started to occur at a $CD59_M$ concentration of approximately 3 μg/ml (Fig. 4). The inhibitory activity of CD59 isolated from erythrocyte membranes ($CD59_E$) was similar to that of $CD59_M$, giving a 73% inhibition at a concentration of 5 μg/ml. Functional activity of $CD59_M$ was tested further by examining whether it bound to the terminal complement complexes. When ^{125}I- labelled $CD59_M$ was mixed with C9DS it bound to the soluble C5b–8 complex during activation of the serum with 4% inulin (Fig. 5). In the controls where inulin was omitted, no binding was observed.

DISCUSSION

The GPI-anchored inhibitor of MAC, protectin (CD59), is widely distributed on endothelial and epithelial cell membranes

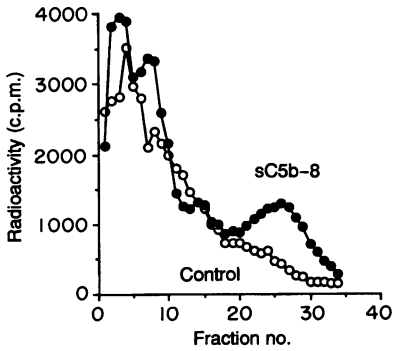

Figure 5. Binding of ^{125}I-labelled $CD59_M$ to terminal complement complexes. Human serum depleted of C9 (60 μl) was activated with 4% inulin in the presence of ^{125}I-labelled $CD59_M$ (90 ng; ●). A control (○) was treated similarly except that no inulin was added. Radioactivity bound to soluble (s)SC5b–8 was separated from free ligand by sucrose density gradient (10–50%) ultracentrifugation. The top of the gradient is to the left.

in the human body.[7] In line with its strong expression in breast duct epithelia,[22] protectin has also been detected in human milk.[18,21] By using immunofluorescence microscopy protectin was found to be strongly expressed on the surface of small globules in the washed cream layer of human milk. It is apparent that these globules represent lipid-rich MFG that are secreted by mammary epithelial cells into milk, rather than degradation fragments of epithelial cells, which would not exhibit similar flotation properties as MFG. The triglyceride core of the MFG is enveloped by a membrane derived from the protectin expressing epithelial cells of the mammary gland. The MFG membrane consists of three zones. The two outer layers are separated from the core by a proteinaceous coat. The narrow middle layer is an apparent unit membrane, while the outermost layer or glycocalyx is rich in carbohydrates and corresponds to a typical glycocalyx of a cell.[27] The CD59-specific staining was unequally distributed over the surface of the MFG, showing areas of intense fluorescence in contrast to darker regions (Fig. 1). The membranes of MFG are unstable, and soon after secretion into alveolar lumen MFG lose some of their membranous material through vesiculation.[2] The inhomogeneous protectin-containing aggregates outside the MFG particles (Fig. 1C) may represent material that has been shed from the membranes.

To examine the molecular characteristics of milk protectin, affinity-purified $CD59_M$ was subjected to SDS–PAGE analysis under reducing conditions. In silver-stained gels, $CD59_M$ was detected as four closely associated bands (19 000–23 000 MW). Distinct bands reacting specifically with the BRIC-229 mAb were also seen in immunoblotting analysis, suggesting that these bands represent the same protein with varying apparent molecular weights. In accordance with earlier studies[28] with erythrocyte CD59, deglycosylation of milk protectin with endo-glycosidase F resulted in a reduction in its apparent molecular weight (Fig. 3). The distinct bands disappeared, leaving a diffuse smear of 14 000–16 000 MW, which apparently corresponds to molecules from which the *N*-linked carbohydrate has been removed. The variability in molecular weight possibly results from heterogeneous glycosylation of $CD59_M$. For CD59 extracted from human urine it has been shown that both the *N*-linked carbohydrate at Asn[18] and the C-terminal GPI anchor display considerable heterogeneity in glycosylation (S. Meri, T. Lehto, C. W. Sutton, V. Tyynelä & M. Baumann, manuscript submitted for publication).[29] A band corresponding in mobility with the non-glycosylated form of $CD59_M$ was also visible in the undigested sample of milk protectin analysed by immunoblotting. The results thus indicate that breast milk contains different variants of protectin.

In addition to breast milk, protectin has been detected in various other human body fluids, including human urine, seminal plasma and amniotic fluid.[6,18,30–32] In seminal plasma and amniotic fluid the principal form of protectin has the glycophospholipid anchor. In urine most of protectin lacks the phospholipid tail of the anchor.[17,29,33] The hydrophobic phospholipid tail gives protectin the ability to incorporate into lipid bilayers. Twenty-nine per cent of the affinity-purified ^{125}I-$CD59_M$ became incorporated into GPE. Under similar conditions only 4% of urinary CD59 became associated with the cells. This suggests that the majority of the $CD59_M$ molecules have an intact glycophospholipid anchor. $CD59_M$ incorporated into GPE protected these cells from subsequent lysis by human complement. The full inhibitory activity of protectin requires the presence of an intact phospholipid tail and therefore the urine-derived soluble CD59 is an ineffective inhibitor of complement lysis.[6,18] A potential drawback, however, for the use of lipid-tailed protectin as a MAC inhibitor is that its incorporation into cell membranes is inhibited by plasma lipoproteins and albumin.[34]

The physiological significance of protectin in milk remains unknown. It could simply represent a GPI-anchored protein that has become sloughed off from the cell membranes during lipid secretion. An intrinsic property of milk in killing and preventing the invasion of pathogens in the gastrointestinal tract of an infant is accomplished mainly by non-inflammatory mechanisms, i.e. lactoferrin, lysozyme, fibronectin and IgA.[1] Components of the complement system and complement-activating immunoglobulins IgG and IgM are present in milk but at low levels.[35] IgA, the most abundant immunoglobulin in milk, does not activate complement and thereby also limits inflammatory responses in the gut.[36] In addition, a number of anti-inflammatory substances, including antioxidants, catalase and histaminase, are present in milk.[36] This may be particularly relevant as during the post-partum period both the infant and the mother are exceptionally prone to infections with associated inflammation. Both the diversity of anti-inflammatory agents in milk and the established role of MAC in inducing sublethal inflammatory responses[37] suggest that the physiological role of milk protectin would be to suppress MAC-mediated tissue damage and inflammation during lactation in both parties involved.

As results of the present study show that functionally active glycolipid-anchored molecules are secreted into milk, this feature might be exploited as a means to produce bioactive molecules that need to be targeted into cell membranes. An obvious example for potential reconstitution therapy with GPI-anchored complement inhibitors is paroxysmal nocturnal haemoglobinuria (PNH), a human disease characterized by a deficiency of GPI-anchored glycoproteins, haemolytic episodes and an increased tendency for vascular thromboses.

To allow large-scale production of protectin, the human protectin gene should be transferred to dairy cattle. Purification of GPI-anchored molecules from milk would be simple since MFG can be easily isolated and a long tradition exists for various separation procedures. Milk could thus serve as a source for effective production of molecules that either inherently possess a GPI anchor or to which the GPI anchor could be tethered by genetic engineering. If appropriate, the molecules could also be solubilized by cleavage with PIPLC.

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