Calreticulin (CRT) is best known as a calcium-binding, endoplasmic reticulum-resident, 60-kDa chaperone. The presence of CRT in the endoplasmic reticulum is due to the KDEL receptor, which interacts with the KDEL-containing C-terminal domain of CRT. In addition to its endoplasmic reticulum location, CRT has been found at other sites including its functional presence at the surface of a variety of cells. Most of these studies utilized cultured cells, such as mouse melanoma cells (1), human fetal fibroblasts (2), HeLa cells (3), in vitro activated T cells (4), bovine aortic endothelial cells (5), CEM, and Jurkat cells (6). Because there is always a fraction of dead and dying cells within a population of cultured cells, it raises the question as to whether the ecto-calreticulin (eCRT) is derived from moribund cells and stuck to the surface of viable cells. However, eCRT has been identified on the surface of freshly isolated human polymorphonuclear neutrophils (PMN) (7, 8), a finding that we have confirmed (9).

Engaging eCRT induces different effects in different cells, e.g. for macrophages, ligation of eCRT with either complement C1q-, mannan-binding lectin-, surfactant protein A-, or surfactant protein D-opsorized apoptotic cells, induces phagocytosis of the apoptotic cell by a macrophage CD91-dependent mechanism (10, 11). For fibroblasts, engaging eCRT by Bβ chain of fibrinogen induces mitosis (9), whereas engaging eCRT by the collagen domain of C1q induces a pro-apoptotic, anti-mitotic effect (12). CRT, which lacks a transmembrane domain, requires an adaptor molecule that is a resident of the plasma membrane to be expressed at the cell surface. The effect of eCRT engagement would then depend to a great extent on the identity of the adaptor molecule(s) of the plasma membrane.

In this study, we report that CRT is present at the plasma membrane of circulating PMN. Normal 293 cells expressed eCRT, whereas GPI-anchor-deficient 293 cells were also eCRT deficient, consistent with the putative adaptor molecule(s) for eCRT being GPI-anchored. Using immunoprecipitation and confocal microscopy, we have identified CD59 as a major adaptor protein for eCRT in PMN. Finally, cross-linking eCRT with primary and secondary antibodies induced a calcium flux in PMN, demonstrating that eCRT ligation with a natural ligand has the potential to initiate intracellular signaling.

EXPERIMENTAL PROCEDURES

Reagents—Reagents were purchased as noted: EDTA, MLEP, BSA, protein A, protease inhibitor mixture, sodium vanadate, Tris, propidium iodide acridine orange, C5a, and MLEP (Sigma); Dextran-70 (McGaw, Irvine, CA); Ficol-Hypaque (Amersham Biosciences); and HBSS with Ca2+ and Mg2+ and without (HBSS−) (Invitrogen). Tris-buffered saline-Tween buffer consisted of 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, Tween 0.1%.

Antibodies—The following antibodies were used: rabbit anti-CRT peptide residues 405–417 (Stressgen, Victoria BC, Canada); chicken anti-CRT peptide residues 339–414 (Affinity Bioreagents, Golden, CO), mouse anti-CRT mAb SPA-601 (Stressgen); rabbit anti-DAF (13); mouse anti-CD11b mAb 3G8 (14); anti-CD59 mAbs VTH 53.1 (15), Bric229, p282, MEM 43/5 (16); anti-CD67 mAb Vim 5 (BD Biosciences);
activity estimation reporter mAb CBR M1/5 for CD11b/CD18 (17); horseradish peroxidase-conjugated goat anti-mouse and -rabbit IgG (Zymed Laboratories Inc., San Francisco, CA); and fluorescently labeled secondary antibodies of "ML grade" (multiple labeling, specifically designed for simultaneous detection of two or more antibodies) and normal goat and human serum for blocking (Jackson Immuno Research, West Grove, PA).

Cytometry—Leukocytes were derived from finger prick blood (150 µl) that was mixed with 1 ml cold HBSS*—2 µl EDTA, the cells pelleted by centrifugation, and the erythrocytes lysed by ammonium chloride (0.1 ml 1 M ammonium chloride, 1.0 g/l potassium bicarbonate, and 0.037 g/l EDTA) for 5 min. The leukocytes were then washed twice in HBSS. Dextran-sequested leukocytes were isolated from 40 ml of ACD-anticoagulated blood obtained by venopuncture, as described (18). PMN were further fractionated from the dextran-leukocyte preparation by centrifugation through Ficoll-Paque at 3000 × g for 20 min. 123 fetal kidney epithelial cells were transfected with an SV40 large T antigen plasmid and exposed to ethyleneminesulfonate. Subsequently, a normal GPI-anchor expressing cell line (293Tag1.6) and a GPI-anchor deficient expressing cell line (A293.2.2) were cloned by limiting dilution.

Cells—Leukocytes were derived from finger prick blood (150 µl) that was mixed with 1 ml cold HBSS*—2 µl EDTA, the cells pelleted by centrifugation, and the erythrocytes lysed by ammonium chloride (0.1 ml 1 M ammonium chloride, 1.0 g/l potassium bicarbonate, and 0.037 g/l EDTA) for 5 min. The leukocytes were then washed twice in HBSS. Dextran-sequested leukocytes were isolated from 40 ml of ACD-anticoagulated blood obtained by venopuncture, as described (18). PMN were further fractionated from the dextran-leukocyte preparation by centrifugation through Ficoll-Paque at 3000 × g for 20 min. 293 fetal kidney epithelial cells were transfected with an SV40 large T antigen plasmid and exposed to ethyleneminesulfonate. Subsequently, a normal GPI-anchor expressing cell line (293Tag1.6) and a GPI-anchor deficient expressing cell line (A293.2.2) were cloned by limiting dilution (19). FPC-1, prostate epithelial cells, were provided by S. Tomlinson (Medical University of South Carolina).

Flow Cytometry—Cells, which were impermeable and alive unless otherwise noted, were incubated for 15 min with antibodies, as noted in each figure, in FACS buffer (HBSS*—5% BSA) at 4 °C, followed by two washes and incubation for 15 min with secondary antibody at a dilution recommended by the manufacturer. Cells were washed once and analyzed in a FACSscan™ (BD Biosciences). In all the experiments at least 10,000 events were recorded, and the results were analyzed using CellQuest Pro 4.0.1.

Immunofluorescence and Confocal Microscopy—All the staining steps were performed in 1.5 ml Eppendorf tubes at 4 °C for 10–15 min in HBSS*—0.5% BSA. Before adding primary and secondary antibodies cell were blocked by incubation with HBSS*—1.5% BSA for 10 min. The final concentration of primary antibodies was 10 µg/ml and the dilutions of secondary antibody were as specified by the manufacturer. After incubation with secondary antibodies, PMN were washed twice and resuspended in HBSS*—1% BSA and incubated with either anti-CD59 (MEM 43/5) mouse or anti-CRT IgG and corresponding control antibodies for 15 min at 4 °C. The cells were washed with cold buffer then warmed to 37 °C for 10 min prior to starting each experiment. The addition of secondary antibody was done while monitoring Ca2+ by measuring Fura-2 fluorescence at 505 nm, using 340/380 nm excitation in a FluoroView F-4500 spectrofluorometer (Hitachi Instruments, San Jose, CA) with constant stirring at 37 °C. The data were analyzed with F4500 Intracellular Cation Measurement software (Hitachi).

RESULTS

Calreticulin Is Bound to CD59 at the Surface of PMN—During a typical isolation procedures the viability of PMN decreases from 100% to about 92–94% as assessed by propidium iodide/acridine orange staining. Dying cells release proteins normally found in the cytoplasm, which can potentially bind nonspecifically to the surface of surrounding cells. This process could explain the presence of an endoplasmic reticulum-resident protein at the cell surface. To test this possibility, we purified PMN and checked them for CRT expression using biotinylated mAb 7.6, which recognizes CRT on the surface of normal PMN. The isolated and stained cells were then analyzed using a FACScan™ (BD Biosciences) equipped with a 15-mW argon laser and a 488-nm excitation wavelength. The 10% of the isolated PMN that were stained for CRT were gated in for analysis. As shown in Figure 1, the PMN population was gated in for analysis (middle). A 1-ml aliquot of the dextran-sequestated cells was used to further purify the PMN by centrifugation through Ficoll-Paque. The pellet that contained more than 95% PMN was analyzed for CRT expression (right). The filled histograms represent control antibody.

Immunoprecipitation and Western Blotting—Isolated PMN were washed twice in HBSS and lysed in lysing buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, and protease inhibitor mixture (Sigma, P 2714) for 30 min on ice. The mixture was centrifuged for 20 min at 15,000 × g, and the post-nuclear supernatant was pre-cleared for 12 h with protein A+G beads (Pierce) for another 4 h with beads-control mAb antibody by end-over-end rotation. The pre-cleared supernatant was then incubated for 4 h with protein A+G beads coupled with either anti-CRT mAb (SPA-601) or anti-CD59 (p282) or anti-CD16 (3G8). Beads were washed in lysis buffer (with Nonidet P-40 0.05%) four times (30 min total) and boiled in non-reducing loading buffer for 5 min. Samples were run on 10% NuPage Bis-Tris gels (Invitrogen), transferred on nitrocellulose paper (Hybond ECL, Amersham Biosciences), and blocked with nonfat dry milk 6% in Tris buffer with Tween 0.1% for 1 h at room temperature. Membranes were incubated with anti-CRT mAb (SPA-601), anti-CD59 (YTH53.1), or anti-CD16 mAb (3G8) for 30 min at room temperature, washed, and incubated with horseradish peroxidase-conjugated appropriate secondary antibody for an extra 30 min. Nitrocellulose membranes were washed extensively in Tris buffer with Tween 0.1% and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to XAR film (Kodak, Rochester, NY).

Spectrofluorometry—Freshly purified PMN were re-suspended in HBSS*—2 µl EDTA, at a concentration of 10⁶ cells/ml and loaded with Fura-2/AM (Molecular Probes) per the manufacturer's instructions for 30 min in HBSS*—room temperature followed by two washes in cold HBSS*—2 µl EDTA. Cells were then re-suspended in HBSS—1% BSA and incubated with either anti-CD59 (MEM 43/5) mouse or anti-CRT mAb and corresponding control antibodies for 15 min at 4 °C. The cells were washed with cold buffer then warmed to 37 °C for 10 min prior to starting each experiment. The addition of secondary antibody was done while monitoring Ca2+ by measuring Fura-2 fluorescence at 505 nm, using 340/380 nm excitation in a FluoroView F-4500 spectrofluorometer (Hitachi Instruments, San Jose, CA) with constant stirring at 37 °C. The data were analyzed with F4500 Intracellular Cation Measurement software (Hitachi).

Calreticulin Is Expressed on the Surface Circulating and Isolated PMN—During a typical isolation procedures the viability of PMN decreases from 100% to about 92–94% as assessed by propidium iodide/acridine orange staining. Dying cells release proteins normally found in the cytoplasm, which can potentially bind nonspecifically to the surface of surrounding cells. This process could explain the presence of an endoplasmic reticulum-resident protein at the cell surface. To test this possibility, we purified PMN and checked them for CRT expression during all the steps. The expression of eCRT increased —10% during the isolation of the cells (Fig. 1), but importantly eCRT was expressed on native PMN derived from finger prick blood (Fig. 1a). CRT lacks a transmembrane do-

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eCRT expression. The interaction between eCRT and PMN has an ionic character but is neither lectin- nor integrin-mediated. a, freshly purified PMN were treated with either HBSS buffer (continuous line) or with HBSS + 100 nM fMLP (dotted line) for 30 min and then analyzed by flow cytometry for the level of eCRT expression. b, freshly purified PMN were treated with either HBSS buffer (continuous line) or with HBSS + 5 mM EDTA (dotted line) for 30 min and then analyzed by flow cytometry for eCRT expression. The signal from control non-immune antibody is represented as filled histograms, c, three-hour-old PMN were treated with either buffer (columns 1 and 2) or buffer with added KCl (0.3 M final) (columns 3 and 4) for the times indicated, and then the supernatant was analyzed by immunoblotting for CRT. The permeability of the cells as assessed by propidium iodide staining, did not change significantly during the incubation with buffer or KCl, as mentioned under "Experimental Procedures."
whose PMN were DAF eCRT. Subsequently, we assessed a more typical PNH patient finding that either CD16 or CD59 might be the adaptor for these results were consistent with, but not prove, the reduced by PIPLC treatment.

GPI-anchored protein on PMN, and its expression was significantly
dotted line, open histogram, incubated with buffer (solid line) and reacted with anti-CD16. CD16 is a known
GPI-anchored protein on PMN, and its expression was significantly
reduced by PIPLC treatment.

Leukocytes normally express sev-
—uria (PNH)-like Patients
Because all GPI-anchored proteins were missing from the mu-
it did not help identify the relevant GPI-anchored protein(s)
d mcexpression was linked to the expression of GPI-anchored proteins, but
failed to express CD59 and failed to express eCRT (Fig. 4, a and c).
These results provided further evidence that eCRT expression was linked to the expression of GPI-anchored proteins, but it did not help identify the relevant GPI-anchored protein(s) because all GPI-anchored proteins were missing from the mutated cells.

eCRT Expression Follows the Expression of CD59 and CD16
on the Affected PMN from Paroxysmal Nocturnal Hemoglobin-
uria (PNH)-like Patients—Leukocytes normally express sev-
geral GPI-anchored proteins on their surface, including CD16, CD59, and CD87 (Fig. 5c), and express eCRT (Fig. 5d). Leuko-
cytes from patients with PNH are characteristically deficient in
the two complement regulatory proteins DAF (CD55) and
CD59 and may be deficient in other GPI-anchored proteins as well. We compared eCRT expression in the PMN from a normal
donor and two PNH-like patients. The first atypical PNH pa-
tient’s PMN were DAF⁻ (data not shown), CD16⁻, CD87⁺, and eCRT was expressed normally (Fig. 5, a and b). These results were consistent with, but did not prove, the finding that either CD16 or CD59 might be the adaptor for eCRT. Subsequently, we assessed a more typical PNH patient whose PMN were DAF⁻ (data not shown), CD16⁺, CD87⁻, and CD59⁺. In this case the PMN were eCRT⁺, consistent with
CD59 being the adaptor and eliminating CD16 as a major
adapter (Fig. 5, e and f). These patient data were confirmed using the prostate epithelial cell line PPC-1, which were
CD59⁻, CD16⁻, and eCRT⁻ with net mean fluorescent channels of 188, 2.3, and 46.5, respectively.

Reciprocal Immunoprecipitation of CRT and CD59—The as-
association of CRT with CD59 expression on the PMN from PMN
patients and PPC-1 cells prompted us to see if there were any
physical interactions between eCRT and CD59. We immuno-
precipitated eCRT using rabbit anti-CRT Ab and probed the samples using either anti-CD59 mAb YTH 53.1 or anti-CD16
mAb. CD59 co-immunoprecipitated with e-CRT, whereas CD16
did not (Fig. 6, upper panels). Performing the reciprocal immu-
noprecipitation with anti-CD59 mAb, we detected eCRT by
immunoblotting in a band of 66 kDa, M₂ (Fig. 6, lower panel), the same M₂ as intracellular CRT (data not shown). Interestingly,
only one anti-CD59 mAb (MEM 43/5) was effective in immuno-
precipitating eCRT. The other anti-CD59 mAbs that failed to
co-immunoprecipitate eCRT, including YTH 53.1 and BRIC229, which bind epitope 1, and p282, which binds epitope
3 (25). These data suggest that e-CRT shields epitopes 1 and 3
of CD59, but not epitope 2.

eCRT Co-localizes with a Fraction of CD59 at the Surface of
the PMN—Using fluorescence microscopy it is possible to de-
tect the presence of two or more proteins in the same location
at the same time (co-localization) in a cell. We used this
approach to investigate the distribution of eCRT and CD59 on
human PMN. Freshly purified cells were incubated in HBSS +
1% BSA with either with rabbit anti-CRT Ab or with rat mAb
anti-CD59 (YTH 53.1) for 10 min on ice followed by two washes
and incubation by Cy-3 anti-rabbit and Cy-2 anti-rat secondary
antibodies. The results demonstrate that eCRT is present at
the surface of PMN (Fig. 7, b–d) and co-localizes with a fraction
of CD59 (Fig. 7f). Similar results were obtained with three
other anti-CD59 mAbs. As a negative control we used mAb
CBR M1/5, which recognizes an activation-specific epitope
of CD11b/CD18 (17) (Fig. 7h). In migrating PMN, most of the
cross-linked proteins (if not all) tend to accumulate at the rear of the cell producing the illusion of co-localization, as is demonstrated in Fig. 7. In additional studies, to avoid any possible false results, cells were fixed while still round and imaged using confocal microscopy followed by deconvolution. A PMN is shown after three-dimensional reconstitution using both red (eCRT) and green (CD59) channels showing all the surface staining (Fig. 8a), and two sections through the middle of the cell, which show rings of staining and prove that the cell was not permeable at the time of labeling (Fig. 8, b and c). Most of the signals came from the same location at the cell surface (Fig. 8d), whereas others were either adjacent or separate (Fig. 8, e and f). All the cells examined had more CD59 on their surface than eCRT, but different donors had different levels of eCRT expression.

**Antibody Cross-linking of eCRT on PMN Induces a Calcium Flux Similar to that Induced by Anti-CD59 Cross-linking—**

Antibody-induced cross-linking of GPI-anchored proteins can stimulate intracellular signaling. The signaling pathway used when CD59 is cross-linked includes a Ca\(^{2+}\)/H\(^{+}\) flux and the activation of tyrosine kinases (26, 27). To test if cross-linking eCRT would induce a signal, cells were loaded with Fura-2/AM for 30 min, washed in cold HBSS\(^{2+}\)/H\(^{+}\), and mixed with either anti-CD59 mAb or anti-CRT IgY for 15 min at 4°C. Subsequently the cells were washed with cold buffer then warmed to 37°C for 10 min prior to addition of secondary antibody. As a negative control cells were treated with non-immune IgY, followed by secondary Ab, and no flux was seen (Fig. 9, bottom). Cross-linking eCRT induced a smaller Ca\(^{2+}\)/H\(^{+}\) flux (Fig. 9, middle) than cross-linking CD59 (Fig. 9, top), and one possible explanation is that there is less eCRT-CD59 for cross-linking than there is total CD59, i.e. (eCRT-CD59 + free CD59) for cross-linking, as noted in the confocal images (Figs. 8, a–c).

**DISCUSSION**

In our systematic search for the adaptor molecule(s) responsible for attaching eCRT to the plasma membrane, we found no evidence that the chemotactic factors fMLP (Fig. 2a) and C5a significantly modified the expression of eCRT on PMN, arguing against CRT being stored in a mobilizable intracellular compartment. Experiments with MBCD and PIPLC supported the existence of GPI-anchored adaptor molecule(s) for eCRT (Fig. 3). Finding that the ethylmethanesulfonate-mutated A293.2.2 cells, which lack GPI-anchors, also lacked eCRT was further evidence that adaptor(s) for eCRT were GPI-anchored (Fig. 4). However, chemical mutagenesis can induce “silent” mutations,
FIG. 7. eCRT partially co-localizes with CD59 in PMN. Freshly purified PMN were incubated with either control Ab (a) or polyclonal anti-CRT Ab (b–f). Additional primary Abs were added as follows: anti-CD59 (g) or mAb that recognizes an activation epitope of MAC-1 (CBR M1/5) (h). Cells were incubated with primary Ab for 10 min at 4 °C. After the incubation cells were washed once in cold HBSS + 1% BSA and incubated with Cy-2 anti-rat and Cy-3 anti-rabbit secondary antibodies for 10 min on ice. Cells were washed once and allowed to adhere to the slides for 7 min and then fixed. DIC Nomarski (a, b, and d) and phase contrast images (e and g) were used either alone or overlaid with fluorescence microscopy images (b and d). Although control Ab showed no significant fluorescence (a), eCRT (b and magnified in d) displayed a punctated distribution clustered at the rear of the cell. A cell co-stained for eCRT and CD59 is shown in phase contrast microscopy (e) and fluorescence (f). The signals from both antibodies were clustered at the rear of the cell. A similar pattern (distribution at one end of the cell) was seen when anti-CD59 and CBR M1/5 mAb were used (h).

FIG. 8. Confocal images of PMN in suspension. PMN were stained as above and fixed in suspension with 3.8% paraformaldehyde for 5 min. Cells were then washed and mounted in anti-fading media on slides. Serial sections were acquired using a confocal microscope and used to reconstitute the whole PMN (a). PMN display a strong surface staining for CD59 (green) and eCRT (red) with no detectable intracellular staining (b and c). There was significant co-localization detailed in d. In b and magnified in e CD59 and eCRT were in adjacent positions, whereas in c and magnified in f they were in separate locations at the plasma membrane.
CD59 was initially described as a complement regulatory protein that interacts with C8 and C9 to inhibit cell lysis by the terminal complement pathway (15, 29–32). It is unknown whether eCRT bound to CD59 inhibits or augments the complement regulatory activity of the latter. Erythrocytes depend heavily on CD59 to protect them from complement-mediated lysis (33), and the fact that they bear minimal levels of eCRT (9) suggests that eCRT is not required for CD59 complement regulatory activity. On the other hand, if the eCRT-CD59 complex inhibits the assembly of the terminal complement components on the cell surface, then eCRT may modulate complement-dependent signaling to the cell (27, 34, 35). The calcium flux that we (Fig. 8) and others (36) have noted when eCRT is cross-linked is consistent with the finding that cross-linking any of the abundant GPI-anchored proteins on PMN (CD16, CD48, CD55, CD58, and CD59) induces a calcium flux (26).

CD91 has been associated functionally with eCRT in human macrophages, initially as a candidate receptor for soluble CRT uptake (37, 38), and additionally as part of a complex with eCRT that functions for the uptake of C1q and collectin-opsonized particles, by their respective collagen domains (10, 11). However, no direct binding of eCRT to CD91 has been demonstrated at date. PMN do not express CD91 and therefore would require a different molecule as their adaptor (39). 293 cells express both CD59 and low levels of CD91, the latter of which is not GPI-anchored (39). In the GPI-anchor-deficient A293.2.2 cells we found that eCRT was also deficient (Fig. 4). Thus, in PMN and in the fetal kidney epithelial cell line 293, CD91 does not serve as an adaptor for eCRT. PIPLC treatment of human monocytes also decreased expression of cell surface CRT (data not shown), and if this were also true for monocyte-derived macrophages, it seems likely that CD91 must participate in a complex with eCRT and a GPI-anchored adaptor.

We have found that CR1 (complement receptor 1, CD35) is a functional phagocytic receptor on PMN and adhesion receptor on erythrocytes for the collagen domain of complement C1q and the homologous opsonin, mannan-binding lectin (18, 40, 41). However, the ability of isolated or recombinant CRT to bind C1q is also well described, giving rise to the name cC1qR (42–45). In equilibrium binding assays, C1q collagen tail binding to purified CRT requires low ionic strength buffer, as does binding to CR1 (40, 46); whereas C1q globular domains bind to adjacent sites in recombinant CRT in normal ionic strength buffer (8). Immobilized C1q induces PMN to make and release superoxide (47, 48). The PMN receptor that mediates this response is not CR1 (48). Others have found that rabbit anti-C1qR partially inhibits C1q-mediated superoxide production by PMN (21), a finding we have confirmed using anti-CRT IgY against a peptide of amino acid residues 24–43 (data not shown). These data suggest that eCRT might be the receptor for this C1q-mediated PMN response.

In our studies eCRT and intracellular CRT had the same mobility on gels. However, rabbit antiserum raised against the C1q-binding protein isolated from plasma membranes ("cC1qR") (42) cross-reacts with calreticulin, but consistently recognizes a band of 68 kDa, whereas calreticulin has a Mr of 60 kDa. Thus, although it seems likely that C1q binds cellular CRT, the relationship between eC1qR and eCRT remains unclear. Although there is a second gene for CRT in humans, its expression is limited to testis, and thus cannot explain any putative differences between intracellular and eCRT in PMN (49).

Finally, the functional consequences of ligand binding to eCRT will depend upon the adaptor proteins engaged. Our identification of GPI-anchored proteins as the primary adap-
tors for eCRT will now provide direction for future studies in this field.

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