RELEASE OF DECAY-ACCELERATING FACTOR (DAF) FROM THE CELL MEMBRANE BY PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C (PIPLC)

Selective Modification of a Complement Regulatory Protein

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Decay-accelerating factor (DAF) is a membrane protein that inhibits amplification of the complement cascade on cell surfaces. By binding to C3b or C4b complement fragments that accidentally deposit on the cell membrane, DAF blocks the assembly of both the classical and alternative C3 and C5 convertases (1–3). In this manner, DAF protects host cells from damage by autologous complement.

DAF is deficient in a rare acquired disorder of blood cells, paroxysmal nocturnal hemoglobinuria (PNH) (4, 5), characterized by increased sensitivity of erythrocytes to complement-mediated lysis (6). The DAF-deficient blood cells probably originate from the expansion of abnormal bone marrow clones (7, 8), but the nature of the molecular defect is unknown. Other membrane abnormalities have been found in PNH, including the absence of acetylcholinesterase (AChE) in the population of complement-sensitive erythrocytes (9).

These two apparently unrelated proteins, DAF and AChE, share the property of being able, in a purified form, to reincorporate into the lipid bilayer of cell membranes or into phospholipid vesicles (3, 10, 11). The amphipathic properties of AChE are known to be due to an unusual type of membrane anchor, the diacylglycerol moiety of a phosphatidylinositol (PI) molecule that is covalently attached to the protein (11, 12). The structure of this membrane-seeking domain was revealed, in part, by the observation that AChE is released from the cell surface by phosphatidylinositol-specific phospholipase C (PIPLC) (13, 14). In view of the above-mentioned similarity between the amphipathic properties of DAF...
and AChE, as well as the fact that they are both absent from a clonal disorder, we studied the sensitivity of DAF to PIPLC.

Materials and Methods

Buffers, Reagents, Complement Components, and Red Cell Hemolytic Intermediates. Buffers for the enzymatic treatments were Dulbecco’s PBS, or Heps-buffered isotonic saline (HBS) (0.154 M NaCl; 10 mM Heps, pH 7.4). In the hemolytic assay, buffers were isotonic veronal-buffered saline (DGVB**) (2.5 mM veronal, pH 7.5; 73 mM NaCl; 2.5% dextrose; 0.15 mM CaCl₂; 0.5 mM MgCl₂; and 0.1% gelatin) and the metal-chelating veronal buffer (GVB-EDTA) prepared as above, but without dextrose, CaCl₂ or MgCl₂, and supplemented with 10 mM EDTA. For hypotonic lysis a phosphate buffer was used (0.005 M sodium phosphate, pH 7.5; 0.001 M EDTA; 50 mM PMSF [reference 2]). C1, C2, C4, and C3-9 (C-EDTA) were prepared as described previously (3). EAC142 cells were prepared from antibody (IgG)-sensitized sheep erythrocytes (EA), by sequentially reacting guinea pig C1 and human C4 and C2 with EA (5).

Phospholipase C (Type I from Clostridium perfringens), BSA (R1A grade), OVA (grade V), PMSF, and soybean trypsin inhibitor (SBTI) were purchased from Sigma Chemical Co., St. Louis, MO. Trypsin was purchased from Cooper Biomedical, Inc., Malvern, PA.

Hemolytic Assay. Inhibition of the classical pathway C3-convertase (C4b2a) was quantitated as described by Nicholson-Weller et al. (2). Briefly, EAC142 cells containing ~1.5 C4b2a hemolytic sites per cell were allowed to decay for 15 min at 30°C in the presence or absence of various concentrations of DAF. Residual C4b2a sites were developed by incubation of the intermediates with C-EDTA for 1 h at 37°C.

Preparation of PIPLC. PIPLC was prepared from Staphylococcus aureus as previously described (15). Briefly, culture supernatant was first centrifuged, the pH adjusted to 5.5, and the supernatant loaded onto a column of Amberlite CG-50 (Sigma Chemical Co.). The column was eluted with a linear salt gradient, the eluate concentrated by ammonium sulfate precipitation (90% saturation), and dialyzed. The concentrate was then chromatographed on Sephadex G-75-preequilibrated in Tris-acetate buffer. Purity as judged by SDS-PAGE was ~90%. The final preparation was lyophilized and stored at 4°C.

Purification of mAbs. Two mAbs were used, IA10 (IgG2a), and IIH6 (IgG1) (8). They were purified from ascites fluids by precipitation with 50%-saturated ammonium sulfate, followed by DEAE-Sephadex chromatography. Polyclonal anti-DAF antibody was prepared by immunization of rabbits with purified DAF.

Two-Site Immunoradiometric Assay (IRMA) for DAF. This was performed essentially as described previously (8). Briefly, plastic plates (96-U-bottomed wells; Becton, Dickinson & Co., Oxnard, CA) were coated with 20 µg/ml of anti-DAF monoclonal IA10 diluted in PBS. The wells were incubated with the DAF-containing extracts, washed three times with PBS containing 1% BSA and 0.02% NaN₃ (AZ), and then incubated with the revealing antibody, 125I-labeled anti-DAF mAb (IIH6). After being washed, the wells were counted. The amount of DAF was calculated by comparison of the values in the samples with the values in the standard curve obtained using purified DAF.

Immunopurification of DAF. 3 mg of purified mAb to DAF, (IA10) or an irrelevant mAb, were coupled to CNBr-activated Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer’s instructions.

DAF was isolated by affinity chromatography from the supernatant of PIPLC-treated human erythrocytes (huE), or from the solubilized extracts of erythrocyte membranes. To remove materials that bound nonspecifically to the beads, the supernatants were first incubated by rotation at room temperature for 1 h with 100–200 µl of sepharose beads bearing the irrelevant mAb; the supernatant was then incubated for an additional 2 h with 50 µl of beads bearing the monoclonal anti-DAF antibody. The beads were washed with 400 µl of PBS containing 0.001% NP-40 and the bound DAF was eluted by incubation for 1 h at room temperature with PBS-0.001% NP-40 containing 0.1 M triethylamine, pH 11.5 (16). The eluate was neutralized by the addition of 1 M Tris-HCl, pH 7.4. DAF purified by this method retained full biological activity.
For the large scale preparation of DAF, the starting materials were detergent extracts from erythrocyte membranes. These were subjected to affinity chromatography as above, and then further purified by HPLC on a TSK-3000 SW gel-filtration column (LKB Produkter, Bromma, Sweden). The final preparations of DAF were >90% pure, as determined by silver staining. Details of this procedure will be published elsewhere (Davitz, M. A., and V. Nussenzweig, manuscript in preparation).

Preparation of Blood Cells. Citrated blood was centrifuged at 400 g for 10 min at 4°C. The plasma and buffy coat were removed and the huE washed four times with PBS before use. huE were stored for up to 1 wk at 4°C after venipuncture. Mononuclear cells were isolated by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals). Neutrophils were obtained by centrifugation of fresh citrated blood on Ficoll-Paque followed by dextran sedimentation (17).

PIPLC Treatment of Cells. huE (10⁹/ml) were suspended in HBS containing 1 mg/ml OVA. PIPLC was then added (concentration specified in the text), and the mixture incubated at 37°C (time specified in the text). After incubation, the cells were pelleted by centrifugation for 30 s and the supernatant removed. The cells were washed twice in the same buffer and incubated for 15 min at room temperature in PBS containing 1% NP-40 with 1 mM PMSF; insoluble material was removed by centrifugation at 12,000 g for 15 min. DAF in both the supernatants and the solubilized membranes from PIPLC treatment was assayed by IRMA.

Mononuclear cells and neutrophils (2–5 × 10⁸ cells/ml) were suspended in PBS containing 1% BSA and 0.1%AZ; PIPLC (final concentration 15 μg/ml) was then added. The cells were incubated for 15 min at 37°C, pelleted by centrifugation, and the supernatant removed. The cells were washed twice with the same buffer and analyzed for the presence of DAF by immunofluorescence using a FACS. Alternatively, DAF was assayed in the supernatant and solubilized pellet by IRMA as described above.

In all experiments duplicate samples were processed and the results represent the mean values. Variation around the mean was always <10%. Negative controls, in which PIPLC was omitted, were included in each experiment.

Reincorporation of ¹²⁵I-labeled DAF into huE (3). Purified membrane DAF or DAF isolated from the supernatants of erythrocytes treated with PIPLC (DAF-S) were labeled to similar specific activities (1–2 × 10⁶ cpm/μg) with ¹²⁵I. 143 ng of ¹²⁵I DAF or DAF-S were incubated with 1.7 × 10⁸ huE for 1 h at 37°C in a volume of 120 μl of HBS containing 1 mg/ml OVA. The cells were overlayed on FCS, pelleted by centrifugation, and then washed three times in HBS containing 1 mg/ml OVA to remove nonspecifically adherent material.

Trypsinization of huE. A suspension of huE in HBS (10¹⁰/ml) mixed with trace amounts of ¹²⁵I surface-labeled huE was incubated for 15 min at 37°C in a volume of 300 μl, either with trypsin (150 μg/ml) or the same concentration of trypsin mixed with 400 μg/ml of SBTI. After incubation, an excess of SBTI was added to the tube containing trypsin alone. The cells were pelleted, the supernatant removed, and the cells washed with HBS. Total cpm released in the supernatant was determined in a gamma counter; DAF released was quantitated by IRMA. Samples of the cells were incubated for 15 min at 37°C, either with PIPLC (15 μg/ml) or buffer alone. The cells were pelleted, and the supernatant and pellet processed as described above to determine the amount of DAF and released radioactive material.

FACS Analysis of Surface DAF. Mononuclear cells and neutrophils were treated with 5 μg/ml of mAb IA10, washed by centrifugation, and then incubated with fluorescein-conjugated sheep F(ab')₂ anti-mouse IgG heavy and light chains (Cappel Laboratories, Cochranville, PA) (8). After washing, the cells were analyzed by FACS. Control cells were treated with irrelevant mAb of the same subclass and then stained as above.

Radiolabeling. Proteins were radiolabeled with ¹²⁵I by Iodogen (Pierce Chemical Co., Rockford, IL). Unbound ¹²⁵I was removed by gel filtration through Sephadex G-25 (Pharmacia Fine Chemicals).

The surface membrane of huE was labeled with ¹²⁵I using Iodogen. Unbound ¹²⁵I was
DAF is Released from huE by PIPLC. huE were incubated with increasing concentrations of PIPLC at 37°C for 1 h. DAF was then assayed both in the supernatant and in the membrane extracts of treated huE by IRMA. DAF was released by the enzyme in a dose-dependent fashion (Fig. 1). In several experiments, a maximum of 10–12% of the total membrane DAF was released at a PIPLC concentration of 15 μg/ml. The amount of released DAF did not increase significantly when the enzyme concentration was 17-fold greater, or when the incubation time was prolonged for up to 3 h. Specificity of the release by PIPLC was shown by the absence of DAF in the supernatants of huE treated with a broad specificity phospholipase C (15 μg/ml) from C. perfringens.

The fact that only 10–12% of the surface DAF was susceptible to release by PIPLC, suggested that there was either a PIPLC-resistant population of DAF molecules on huE, or that some of the membrane DAF was not accessible to the enzyme. To distinguish between these possibilities, we reincorporated purified 125I-labeled DAF into huE and then treated the cells with the enzyme as above. Only 15.3% of the reincorporated DAF counts were specifically released by PIPLC.

We also pretreated huE with trypsin to remove part of the surface molecules that could potentially interfere with PIPLC activity, and then measured the release of DAF by this enzyme. To facilitate interpretation of the results, a small number of 125I surface-labeled huE were added to the bulk of unlabeled eryth-
Table 1

Effect of Trypsinization of Human Erythrocytes on the Release of DAF

| Primary treatment | DAF released | cpm released | Secondary treatment | DAF released | cpm released |
|-------------------|--------------|--------------|---------------------|--------------|--------------|
|                   | ng | % | | ng | % |
| Trypsin           | 1.1 | 28.7 | PIPLC | 5.1 | 0.2 |
| Trypsin + SBTI    | 0.0 | 2.7 | PIPLC | 5.6 | 0.9 |

* Determined by IRMA.

Figure 2. FACS analysis of PIPLC-treated lymphocytes and monocytes. Peripheral blood mononuclear cells isolated on Ficoll-Paque were treated with PIPLC (15 μg/ml). The cells were treated with a single anti-DAF monoclonal, IA10, and then stained with fluorescein-conjugated sheep F(ab')2 anti-mouse IgG. A nonrelevant monoclonal of the same subclass was used as a negative control. (A) lymphocytes; (B) monocytes. Solid lines, fluorescence of cells treated with anti-DAF mAb; dashed lines, fluorescence of cells treated with a control mAb. The mean fluorescence intensities of lymphocytes and monocytes treated with antibodies to DAF were 181 and 410 (left), respectively; after PIPLC treatment (right) the mean fluorescence intensities were: lymphocytes, 33.7 and monocytes, 132.7. The fluorescence intensity of the control cells did not change significantly after PIPLC treatment.

rocytes. The mixture of labeled and unlabeled cells was first treated with trypsin or with trypsin inactivated by SBTI, as described in the Materials and Methods section. Trypsin released 26% of the surface cell-associated counts (Table 1), but only 1.1 ng of DAF (~2% of the total surface DAF). The trypsin-treated and control cells were then incubated with PIPLC (15 μg/ml) for 15 min at 37°C. The specific release of labeled surface molecules by PIPLC did not differ significantly in the two groups and was <1%. There was no difference in the amounts of DAF released by PIPLC in cells treated with trypsin or controls, i.e., 5.1 ng vs. 5.6 ng, which corresponds to ~10% of the surface DAF.

Release of DAF from Lymphocytes and Neutrophils by PIPLC. Next we studied the effect of PIPLC on DAF from the surface of peripheral blood mononuclear cells and neutrophils. The cells were incubated with 15 μg/ml of PIPLC for 15 min at 37°C, and DAF assayed by IRMA in both the supernatants and extracts of membranes from the treated cells. The amount of DAF released by PIPLC corresponded to 80 and 53% of the total DAF that could be extracted from mononuclear cells and neutrophils, respectively. To determine the proportion of membrane DAF that was removed by PIPLC, we subjected the cells to FACS analysis after treatment with mAbs to DAF, followed by staining with fluorescein-
conjugated sheep F(ab')₂ anti-mouse IgG. The mean fluorescent intensities of lymphocytes and monocytes decreased by ~80% (Fig. 2A) and 70% (Fig. 2B), respectively, in the PIPLC-treated cells as compared with controls in which the primary antibody was a nonrelevant mAb of the same subclass. Neutrophils were incubated with 30 μg/ml of PIPLC at 37°C for 15, 30, and 45 min (Fig. 3, B–D, respectively). The decrease in fluorescence intensity reached a plateau of ~60% after 30 min (Fig. 3E). PIPLC had no effect on complement receptor 1 (CR1) of lymphocytes or erythrocytes as determined by FACS analysis using mAbs to CR1 for the primary antibody (data not shown). Additionally, with respect to erythrocytes there was no evidence by FACS of a distinct subpopulation of PIPLC-sensitive huE (data not shown).

Properties of Released DAF. To examine the effect of PIPLC on the structure of DAF, huE were treated with PIPLC and the released material (DAF-S) was purified from the supernatants by affinity chromatography. DAF-S migrated on SDS-PAGE as a single band with an Mᵣ of 67,000, slightly smaller than that of untreated DAF (Fig. 4, lane 2). By Western blotting we confirmed that the smaller molecule was indeed DAF (Fig. 4, lane 4).

We then compared the ability of native DAF and DAF-S to accelerate the decay of preformed C5-convertase. EAC142 cells, with 1.5 hemolytic sites/cell, were incubated with increasing concentrations of DAF-S or DAF. After incubation at 30°C, the cells were washed and residual C₄b₂a sites developed with C-EDTA. As shown in Fig. 5, DAF inhibited C₄b₂a sites in a dose-dependent
FIGURE 4. Effect of PIPLC on the structure of DAF. DAF-S was isolated by affinity chromatography from the supernatants of PIPLC-treated huE. Purified DAF and DAF-S were labeled to similar specific activities with $^{125}$I and subjected to SDS-PAGE followed by radioautography. Lane 1, DAF; lane 2, DAF-S. Unlabeled, purified DAF and DAF-S were subjected to SDS-PAGE, followed by Western blotting using a polyclonal rabbit anti-DAF antibody; lane 3, DAF; lane 4, DAF-S. DAF-S is slightly smaller than DAF. The band with 150,000 Mr shown in lane 3 is DAF-II, a recently discovered form of red cell DAF (Kinoshita, T., S. I. Rosenfeld, and V. Nussenzweig, manuscript in preparation).

FIGURE 5. Comparison of DAF and DAF-S in their ability to accelerate the decay of the C3 convertase, C4b2a. Percent inhibition of the hemolytic activity ($Z$) of the C3 convertase, C4b2a, is plotted against increasing DAF concentration. At 1 ng/ml DAF inhibited $\sim$20% of the C4b2a sites, while DAF-S had no effect at a concentration of 20 ng/ml.

fashion, while DAF-S showed no significant inhibitory effect. The PIPLC treatment also affected the ability of DAF to reincorporate into the cells. In these experiments huE were incubated with purified $^{125}$I-labeled DAF or DAF-S. The cells were washed, the membranes solubilized in sample buffer, and then subjected to SDS-PAGE followed by radioautography. As shown, only native DAF reincorporated into the membrane (Fig. 6, lane 2).

Discussion

A new family of proteins anchored to the membrane by PI has recently been identified; the group includes AChE (11-14), alkaline phosphatase (20), 5'-nucleotidase (21, 22), Thy-1 (23, 24), the variant surface glycoprotein of African trypanosomes (VSG) (25, 26), a merozoite surface antigen of Plasmodium falciparum (27), and a major membrane protein of Leishmania parasites (M. J. Turner, personal communication). The role of PI in the hydrophobic attachment of these
proteins to the cell surface has been demonstrated by enzymatic studies showing their release from the membrane by treatment of the cells with PIPLC, as well as by chemical analysis of the membrane anchors of several of these proteins (28). The detailed analysis of the membrane-anchoring domain of VSG, shows that it is attached via the carboxyl terminus to ethanolamine, which is linked sequentially to an oligosaccharide (29, 30) and glucosamine; the protein-carbohydrate complex is anchored to the cell surface by PI (26). The evidence to date strongly suggests that the other proteins anchored by this mechanism are attached to PI by similar structures on their carboxyl terminus (28).

Here we present evidence that DAF's membrane anchor is PI. DAF is released from the membrane of peripheral blood red cells and leukocytes by PIPLC. The release is highly selective, since treatment with a broad-specificity phospholipase C or with trypsin was ineffective. Furthermore, while PIPLC released <1% of the counts from $^{125}$I-labeled erythrocytes, it removed 10–12% of the cell surface DAF. PIPLC released 70–80% of membrane DAF from peripheral blood mononuclear cells and 60% of the DAF from the surface of neutrophils.

The reason for resistance of part of the surface DAF to PIPLC is not known. Although only ~10% of AChE from human red cells is released by PIPLC (31), available chemical evidence suggests that the protein is anchored exclusively by PI (12). Perhaps PIPLC does not have access to all the PI-anchored DAF molecules. This does not appear to be the case for DAF, since removal of part of the red cell surface proteins by trypsinization before treatment with PIPLC did not increase the release of DAF. More importantly, only 15% of the red cell-purified, radiolabeled DAF that had been reincorporated into the membrane
was released by PIPLC. On the basis of the reasonable assumption that the incorporated molecules are randomly distributed within the erythrocyte membrane and therefore equally accessible to PIPLC, it appears that red cell DAF is structurally heterogeneous. For example, part of the red cell DAF molecules may be secondarily modified on the membrane, rendering them resistant to PIPLC, or alternatively, a portion of the DAF molecules may not be anchored by PI.

The finding that DAF and AChE share similar, if not identical, membrane-spanning domains is relevant to the pathogenesis of PNH. The absence of these two otherwise functionally unrelated proteins in the affected cells from PNH patients suggests that the molecular defect in this disease may lie in the biosynthetic pathways leading to attachment of PI to the polypeptide chain, in the transport of these proteins to the surface membrane, or in their release by the action of endogenous phospholipases. According to this hypothesis, other membrane molecules, e.g., alkaline phosphatase, and 5'-nucleotidase, that are anchored by PI (20-22), should also be deficient in PNH cells. In fact, Craddock et al. reported that the levels of leukocyte alkaline phosphatase are decreased in the patient's leukocytes (32).

By SDS-PAGE, DAF-S is slightly smaller than the membrane form. DAF and DAF-S cannot be distinguished antigenically; however, DAF-S has lost the ability to significantly enhance the decay-dissociation of the C3 convertase, C4b2a, as well as its ability to reincorporate into cell membranes. On the basis of prior evidence showing that DAF can only inhibit C3-convertase activity endogenously within the same cell membrane (3), it is likely that the lack of inhibitory activity of DAF-S is causally related to its inability to insert into membranes.

The functional significance of the PI anchor is not known. It has been suggested that it may be energetically more favorable than a membrane-spanning hydrophobic polypeptide sequence (28). We speculate that the PI anchor may increase the rate of lateral mobility of the attached polypeptides in the lipid bilayer. This would be advantageous in the case of DAF, which must move freely and rapidly in the membrane to encounter the randomly distributed C3b and C4b deposited fragments and inhibit their hemolytic activity (3). Further studies using the fluorescence photobleaching recovery method (33) to measure the rates of lateral diffusion of the PI-anchored protein molecules are necessary to substantiate this hypothesis.

From a practical standpoint, the specific release of DAF by PIPLC could provide a novel approach for altering the complement sensitivity of cells. Autologous bone marrow transplantation depends on the elimination of tumor cells from the marrow (34, 35). Classically, leukemic cells are eliminated by successive rounds of treatment of the marrow with mAb and rabbit complement. This method results in >99% removal of residual tumor cells; nonetheless, pretreatment with PIPLC may amplify the effects of the cascade on the surface of antibody-sensitized cells.

Summary

Decay-accelerating factor (DAF) is a 70,000 M, membrane protein that inhibits amplification of the complement cascade on the cell surface, and protects cells
from damage. Purified DAF can be reincorporated into the membrane of red cells and is functional. DAF is deficient in paroxysmal nocturnal hemoglobinuria (PNH), a disease characterized by increased sensitivity of erythrocytes to complement lysis.

We show here that DAF is part of a newly described family of membrane proteins anchored to the lipid bilayer by means of phosphatidylinositol (PI). Treatment with PI-specific phospholipase C (PIPLC) releases 70–80, 60, and 10% of cell surface DAF from mononuclear cells, neutrophils, and erythrocytes, respectively. The PIPLC-released DAF (DAF-S) is slightly smaller (67,000 Mr) than the membrane form. DAF and DAF-S cannot be distinguished antigenically. Furthermore, DAF-S has lost its ability to significantly inhibit the C3-convertase, as well as its ability to incorporate into cell membranes. Since DAF can only inhibit C3-convertase endogenously, i.e., within the membrane of the same cell, it is likely that the loss of activity of DAF-S is causally related to its inability to reincorporate in the lipid bilayer. As shown by others, the complement-sensitive red cells from PNH patients lack acetylcholinesterase, which is also anchored to the membrane by PI (9). Thus it is possible that the molecular defect in PNH lies in the biosynthetic pathways leading to the attachment of PI to the polypeptide chains, in the transport of these proteins to the surface, or in their release by the action of endogenous phospholipases.

From a practical standpoint the specific release of DAF by PIPLC could facilitate killing of tumor cells by amplifying the effects of the complement cascade on the surface of antibody-sensitized cells.

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