Articles

Decay Accelerating Factor of Complement Is Anchored to Cells by a C-Terminal Glycolipid†

M. Edward Medof,*,‡ Elizabeth I. Walter, William L. Roberts,§ Robert Haas,‖ and Terrone L. Rosenberry
Departments of Pathology and Pharmacology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106
Received May 23, 1986; Revised Manuscript Received August 4, 1986

ABSTRACT: Membrane-associated decay accelerating factor (DAF) of human erythrocytes (E^{hu}) was analyzed for a C-terminal glycolipid anchoring structure. Automated amino acid analysis of DAF following reductive radiomethylation revealed ethanolamine and glucosamine residues in proportions identical with those present in the E^{hu} acetylcholinesterase (AChE) anchor. Cleavage of radiomethylated 70-kilodalton (kDa) DAF with papain released the labeled ethanolamine and glucosamine and generated 61- and 55-kDa DAF products that retained all labeled Lys and labeled N-terminal Asp. Incubation of intact E^{hu} with phosphatidylinositol-specific phospholipase C (PI-PLC), which cleaves the anchors in trypanosome membrane form variant surface glycoproteins (mfVSGs) and murine thymocyte Thy-1 antigen, released 15% of the cell-associated DAF antigen. The released 67-kDa PI-PLC DAF derivative retained its ability to decay the classical C3 convertase C4b2a but was unable to membrane-incorporate and displayed physicochemical properties similar to urine DAF, a hydrophilic DAF form that can be isolated from urine. Nitrous acid deamination cleavage of E^{hu} DAF at glucosamine following labeling with the lipophilic photoreagent 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) released the [^{125}I]TID label in a parallel fashion as from [^{125}I]TID-labeled AChE. Biosynthetic labeling of HeLa cells with [^{3}H]ethanolamine resulted in rapid ^{3}H incorporation into both 48-kDa pro-DAF and 72-kDa mature epithelial cell DAF. Our findings indicate that DAF and AChE are anchored in E^{hu} by the same or a similar glycolipid structure and that, like VSGs, this structure is incorporated into DAF early in DAF biosynthesis prior to processing of pro-DAF in the Golgi.

The decay accelerating factor (DAF)[1] is an inhibitor of C3 and C5 convertases, the amplification enzymes of the complement cascade (Hoffman, 1969; Nicholson-Weller et al., 1982; Pangburn et al., 1983a; Medof et al., 1984). It is present in the membranes of all cell types in intimate contact with the complement system (Kinoshita et al., 1985; Nicholson-Weller et al., 1985; Asch et al., 1985). It functions *intrinsically* to block autologous complement activation on cell surfaces (Medof et al., 1984) and thereby prevents injury to host cells which could result from autologous complement attack or from its activation in their vicinity.

DAF isolated from human erythrocytes (E^{hu}) is a single-chain polypeptide with apparent molecular weight of ~70K (Nicholson-Weller et al., 1982; Pangburn et al., 1983a; Medof et al., 1984; Kinoshita et al., 1985). When added to cells in vitro, purified E^{hu} DAF reincorporates into cell membranes (Medof et al., 1984). The uptake is not receptor mediated but rather involves direct interaction between a hydrophobic domain of E^{hu} DAF and the membrane lipid bilayer. A contrasting soluble DAF form, ~3K smaller in apparent molecular weight than E^{hu} DAF, can be isolated from urine (Medof et al., 1985a). The urine DAF is markedly less hydrophobic than E^{hu} DAF and does not incorporate into cells. Hemolytic assays have shown that urine DAF cannot function intrinsically like E^{hu} DAF but can inhibit convertases *extrinsically* (Medof et al., 1985a) in a fashion similar to the serum regulatory factor C4 binding protein (C4bp).

In paroxysmal nocturnal hemoglobinuria (PNH), variable proportions of blood cells are abnormally sensitive to autologous complement [reviewed in Rosse & Parker (1984)]. Affected blood elements accumulate C3b fragments and are damaged in vivo. It has been shown that affected cells are deficient in DAF (Pangburn et al., 1983a,b; Nicholson-Weller et al., 1983) and that the absence of DAF is causally related to the excessive C3b uptake that characterizes the disease

† This investigation was supported by grants from the Arthritis Foundation, the Systemic Lupus Erythematosus Society, the American Diabetes Association, and the Muscular Dystrophy Association and by Grants AI-23507 and NS-16577 from the National Institutes of Health. This work was presented in part at the annual meeting of the Federation of American Societies for Experimental Biology (Medof et al., 1986).
* Address correspondence to this author at the Department of Pathology, Case Western Reserve University School of Medicine.
‡ Investigator of the Arthritis Foundation.
§ Medical Scientist Predoctoral Trainee supported by NIH Grant T32 GM07250.
‖ Supported by a fellowship from the American Heart Association, Northeast Ohio chapter.

[1] Abbreviations: DAF, decay accelerating factor; PNH, paroxysmal nocturnal hemoglobinuria; E^{hu}, human erythrocyte(s); AChE, acetylcholinesterase; PI, phosphatidylinositol; PC, phosphatidylcholine; PI-PLC and PC-PLC, PI- and PC-specific phospholipase C; VSGs, trypanosome variant surface glycoproteins; mfVSGs and sVSGs, membrane form and soluble VSGs; PBS, phosphate-buffered saline; $DGVB^{2+}$, GVB^{2+}, and GVBE, isotonic, isoionic, and EDTA-containing veronal gelatin buffers; NP40, Nonidet P-40 detergent; CR1, complement receptor type 1; E^{sh}, sheep erythrocytes; A, anti-sheep hemolysin; $E^{sh}A$, $E^{sh}AC1$, $E^{sh}AC14$, and $E^{sh}AC142$, E^{sh} intermediates bearing the respective complement components; C4b2a, classical pathway C3 convertase; C4bp, C4 binding protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s); TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/86/0425-6740$01.50/0

(Medof et al., 1985b). Recent studies have demonstrated, however, that the DAF deficit does not account for other membrane alterations present in affected cells [reviewed in Rosse & Parker (1984)], suggesting that it must arise as part of a more general defect (Medof et al., 1985c).

Among the abnormalities in affected PNH E^{hu} is deficiency of acetylcholinesterase (AChE) (Herz & Kaplan, 1973; Rosse & Parker, 1984; Medof et al., 1985c), an amphipathic protein that can be converted by papain cleavage near its C-terminus into a soluble AChE form (Dutta-Choudhury & Rosenberry, 1984). The soluble AChE derivative resembles urine DAF in that it appears 2 kDa smaller than native E^{hu} AChE and no longer can associate with phospholipid vesicles (Dutta-Choudhury & Rosenberry, 1984; Kim & Rosenberry, 1985; Haas et al., 1986). Analyses of the C-terminal AChE fragment generated by this cleavage have revealed the presence of a covalently linked glycolipid rather than hydrophobic amino acids typical of a conventional membrane polypeptide anchor. This non-amino acid anchoring structure is comprised partially of fatty acids, inositol, glucosamine, and ethanolamines (Haas et al., 1986; Roberts & Rosenberry, 1986b). Similar C-terminal glycophospholipid anchoring structures have been described in membrane form variant surface glycoproteins (mfVSGs) of trypanosomes (Holder, 1983; Ferguson et al., 1985a,b) and in Thy-1 surface antigen on murine thymocytes (Tse et al., 1985). The mfVSG and Thy-1 structures include phosphatidylinositol (PI) which is susceptible to cleavage by PI-specific phospholipase C (PLC) (Ferguson et al., 1985b; Low & Kincade, 1985). In mfVSGs, the PI is linked through an oligosaccharide to an ethanolamine that becomes covalently attached to the C-terminus of VSG polypeptide during a posttranslational modification (Holder, 1983).

In the present study, we demonstrate that E^{hu} DAF exhibits many features that indicate such an anchor. Our findings provide further information about this recently discovered class of anchoring structures for membrane proteins which is not only of relevance for PNH but is also of general biological interest.

Materials and Methods

Buffers, Proteins, and Reagents. Phosphate-buffered saline (PBS) contained 150 mM NaCl and 10 mM sodium phosphate, pH 7.4. Isoionic veronal buffer (GVB^{2+}) consisted of 145 mM NaCl, 2.5 mM sodium veronal, 0.1% gelatin, 0.5 mM $MgCl_2$, and 0.1 mM $CaCl_2$, pH 7.3. E^{hu} AChE was extracted and purified by affinity chromatography (Rosenberry & Scoggin, 1984). Rabbit anti-sheep erythrocyte (E^{sh}) hemolysin was from Gibco (Grand Island, NY). Guinea pig C1 and C3–9 (Nelson et al., 1966); human C4 (Tack et al., 1980), C2 (Kerr, 1980), C4bp (Nussenzweig & Melton, 1980), and C3b/C4b receptor (CR1) (Iida et al., 1982); murine monoclonal anti-CR1 antibodies 44D, 57F, and 31D (Iida et al., 1982) and anti-DAF antibodies IA10, IIH6, and VIIIA7 (Kinoshita et al., 1985); and rabbit anti-glycophorin antibodies (Medof et al., 1984) were prepared as described. Purified PI-PLC was a gift of Dr. M. Low, Oklahoma Medical Research Foundation, Oklahoma City, OK. Phosphatidylcholine (PC) specific PLC (Boehringer), phospholipase A2 (Sigma), TPCK–trypsin (Sigma), Triton X-100 (Sigma), Nonidet P-40 detergent (NP40) (Particle Data), Bio-Gel (Bio-Rad), and Sepharose derivatives (Pharmacia) were obtained commercially.

Analytical Procedures. SDS–PAGE on gradient (Rosenberry & Scoggin, 1984) and 7.5% linear slab gels (Medof et al., 1984) was conducted as described. Gels of ^3H- or ^{35}S-labeled samples were dehydrated with dimethyl sulfoxide and impregnated with 2,5-diphenyloxazole followed by water prior to drying. Autoradiography and fluorography were performed at −70 °C on X-Omat XAR-5 film (Kodak). For Western blot analysis, proteins were transferred to nitrocellulose following SDS-PAGE under nonreducing conditions (Kinoshita et al., 1985). Amino acid analysis combined with identification of radiomethylated components was conducted as in Haas and Rosenberry (1985). Stokes radii were estimated by chromatography on Sepharose CL-4B as in Rosenberry and Scoggin (1984).

Affinity Purification of DAF. Outdated E^{hu} (10 units) were (individually) depleted of plasma and residual buffy coat cells by repeated washing with PBS containing 10 mM EDTA and lysed by addition of 30 volumes of 1 mM sodium phosphate, pH 7.4, containing 10 mM EDTA. The resulting stroma were concentrated in a Millipore Pellicon system and extracted for 18 h at 0 °C with 1% NP40, 10 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride in PBS (2.5 mL/10^{10} E^{hu}). After 10-fold dilution with the extraction buffer minus NP40 and centrifugation at 10000*g* for 60 min, the supernatant was dialyzed against 150 mM NaCl/50 mM Tris, pH 7.4, and applied to an affinity column composed of anti-DAF monoclonal antibody IA10 coupled to CNBr–Sepharose and cross-linked with glutaraldehyde (50 mL of extract/5 mg of antibody) which was preequilibrated in the same buffer. After the column was washed with 10 volumes of 0.1% NP40, 0.5 M NaCl, and 0.1 M Tris, pH 7.4, DAF was eluted with 0.1% NP40, 0.05 M triethylamine, and 50 mM Tris, pH 11.2 (Hsiung et al., 1982), and immediately neutralized by collection of 0.5-mL fractions into 0.1 mL of 0.1% NP40 and 0.5 M Tris, pH 6.0, saturated with glycine. Each fraction was assayed for DAF antigen by two-site radioimmunometric assay (see below). Positive fractions were examined by SDS–PAGE and silver staining, and fractions giving characteristic E^{hu} DAF band(s) (Kinoshita et al., 1985) only were pooled, dialyzed against 0.1% NP40 in PBS, and frozen in aliquots at −70 °C. The overall yield of purified E^{hu} DAF was ~3 mg (40%). The product was free of glycophorin and other protein contaminants as assessed by Western blotting using anti-glycophorin antibodies and by SDS–PAGE of ^{125}I-Iodogen-labeled DAF followed by radioautography (Medof et al., 1984).

Radiolabeling. E^{hu} DAF (50 μg/mL in PBS and 0.0006% NP40) (Kinoshita et al., 1985), anti-DAF monoclonal antibody IIH6 (1 mg/mL in PBS), and intact E^{hu} (1×10^9/mL in PBS) were iodinated with ^{125}I using Iodogen (Pierce) according to the manufacturer's instructions. Iodinated proteins and cells were purified by gel filtration followed by extensive dialysis and by repeated washing, respectively. E^{hu} DAF (40 μg/mL in 0.1% NP40 and 20 mM sodium phosphate, pH 7) was radiomethylated with 10 mM $H^{14}CHO$ and 50 mM $NaCNBH_3$ at pH 7 for 15 min at 37 °C (Haas & Rosenberry, 1985). The radiomethylated product was dialyzed extensively. E^{hu} DAF and AChE (60 μg/mL in 0.1% Triton X-100 and 20 mM sodium phosphate, pH 7) were labeled with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) (60–150 μCi; Amersham, 10 Ci/mmol) by photolysis at 350 nm for 15 min as in Roberts and Rosenberry (1986a), and the product was dialyzed extensively. Labeled detergent was removed from [^{125}I]TID-labeled E^{hu} AChE by affinity chromatography (Roberts & Rosenberry, 1986a) and from [^{125}I]TID-labeled DAF by two cycles of chromatography on Sepharose CL-6B (1.5 × 75 cm) in 0.1% Triton X-100 and 20 mM sodium phosphate, pH 7.

Radioimmunometric and Hemolytic Assays and Phenyl-Sepharose Binding. DAF concentrations were evaluated by

Table I: Relative Amounts of Amine Components Labeled by Reductive Methylation of E^{hu} DAF and AChE[a]

sample	n	residues methylated per polypeptide			
		N-terminus	Lys	ethanolamine	glucosamine
E^{hu} DAF	10	0.89 ± 0.05	18.05	1.76 ± 0.07	0.84 ± 0.04[b]
E^{hu} DAF after papain digestion	1	1.05	18.05	0.00	0.01
E^{hu} AChE	6	1.00 ± 0.05	7.40 ± 0.20	1.35 ± 0.06	0.98 ± 0.04

[a] Purified proteins were radiomethylated and, where indicated, digested with papain resin and reisolated. Radiolabeled amines were identified as in Figure 1, and moles of residues methylated were determined from the methyl group specific activity [see Haas & Rosenberry (1985)]. Moles of DAF polypeptide were estimated from the amino acid content of two samples (100–200 pmol) of DAF with an assumed polypeptide mass of 44 kDa.[2] The data for DAF were normalized to the mean lysine content (18.05 ± 2.85 residues) of these two samples. The data for AChE were taken from Haas et al. (1986). Data presented are averages of n analyses. [b] $n = 6$.

a sandwich assay employing anti-DAF monoclonal IA10 for antigen trapping and ^{125}I-labeled monoclonal IIH6 (directed against a different DAF epitope) for antigen quantitation (Kinoshita et al., 1985).

Hemolytic assays were performed as previously described (Medof et al., 1984) using washed E^{sh}AC14 prepared with 10 site-forming units of C4, and E^{sh}AC142 prepared by incubating E^{sh}AC14 with sufficient C2 to yield cells bearing 1 hemolytic site of C4b2a per cell after washing and decay in GVB^{2+} for 15 min at 30 °C (Nicholson-Weller et al., 1982; Medof et al., 1984). Incubations of DAF forms with intermediates were performed at 30 °C in GVB^{2+}.

The interaction of DAF forms with phenyl-Sepharose was studied by using previously described conditions for binding of E^{hu} DAF to the resin (Nicholson-Weller et al., 1982). One hundred microliters of E^{hu} or urine DAF (500 ng/mL in PBS and 0.1% NP40) was incubated at 0 °C for 15 min with 20 μL of phenyl-Sepharose in the same buffer, and after centrifugation, unbound antigen was quantitated by radioimmunometric assay.

Papain Digestion. Radiomethylated E^{hu} DAF (0.4 μg, 80000 ^{14}C cpm) was digested with Cys-activated papain conjugated to Sepharose CL-4B (1 mg on 0.5 mL of packed resin) in 0.1% Triton X-100 and 20 mM sodium phosphate, pH 7 (2.5 mL), for 6 h at 4 °C (Dutta-Choudhury & Rosenberry, 1984). The digestion was terminated by centrifugation, and the supernatant and resin wash were applied to a Sepharose CL-6B column equilibrated as above. A 6-mL peak fraction (12000 ^{14}C cpm) from the labeled peak (30000 ^{14}C cpm) that corresponded to an approximate Stokes radius of 6 nm was selected for analysis.

Nitrous Acid Deamination. E^{hu} DAF and AChE in 0.1% NP40 and 0.1% Triton X-100, respectively, were mixed with trace amounts of the corresponding [^{125}I]TID-labeled proteins, adjusted to 125 mM $NaNO_2$ and 25 mM sodium citrate (pH 4.0), and incubated for 1–4 h at 37–50 °C (Shively & Conrad, 1976). The reactions were terminated by raising the pH to 7 with sodium phosphate buffer.

Biosynthetic Studies. Semiconfluent (60%) HeLa cell monolayers were established in 60-mm tissue culture plates by addition of 10^6 cells in 5 mL of Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and culturing for 18 h at 37 °C in 5% CO_2. Prior to initiation of experiments, cells were incubated 1–2 h with fresh DMEM FBS and then preincubated 1 h with 2 mL of either RPMI or Cys-free RPMI containing 10% dialyzed FBS (Gibco). [^{3}H]Ethanolamine (66 μCi) and [^{35}S]Cys (50 μCi) (New England Nuclear) were added in 100-μL aliquots to cells in complete and Cys-free medium, respectively, and the plates were placed at 37 °C. After various times, the plates were transferred to ice, the medium was removed, and the monolayers were washed and extracted with 250 μL of 2% SDS containing 100 units/mL trasylol (Mobay Chemical Corp., New York, NY) using a rubber policeman. The extracts were boiled for 10 min, diluted 5-fold with 50 mM Tris-HCl, pH 7.4, and 190 mM NaCl containing 100 units/mL trasylol, 6 mM EDTA, and 2.5% Triton X-100, and clarified by centrifugation at 10000*g* for 15 min. Prior to immunoprecipitation, extracts were preadsorbed with 100 μL of protein A–Sepharose (10%) for 2 h at 20 °C and centrifuged. Supernatants were transferred to new tubes, pooled anti-DAF monoclonals IA10, IIH6, and VIIIA7 (5 μg/mL each) or pooled nonrelevant monoclonals (of the same Ig subclasses) were added, and the mixtures were rotated for 2 h at 20 °C. Immune complexes were precipitated by addition of 100 μL of fresh protein A–Sepharose (10%), further rotation for 1 h at 20 °C, and centrifugation. The beads were transferred to new tubes and washed twice with the 50 mM Tris-HCl buffer described above, and immunoprecipitated proteins were extracted by addition of 50 μL of SDS–PAGE sample buffer followed by boiling for 10 min.

Results

Identification of Ethanolamine and Glucosamine in Small C-Terminal Fragments of E^{hu} DAF. One feature of the C-terminal glycolipid anchor of E^{hu} AChE is the presence of ethanolamine and glucosamine residues with free amino groups that can be radiomethylated (Haas et al., 1986) and identified following protein hydrolysis by coelution of their mono- and diradiomethylated derivatives with radiolabeled standards in an amino acid analyzer (Haas & Rosenberry, 1985). To determine if these components are also available for radiomethylation in E^{hu} DAF, purified E^{hu} DAF was treated with $H^{14}CHO$ and $NaCNBH_3$ and the product subjected to automated amino acid analysis.

An elution profile of labeled components of radiomethylated E^{hu} DAF is shown in Figure 1, and the results are compared with those of AChE in Table I. Most of the label was incorporated into N^{ϵ},N^{ϵ}-dimethylated Lys, indicating that 18.1 Lys per DAF polypeptide were accessible to methylation under the nondenaturing conditions. Virtually the only major N^{α}-radiomethylated amino acid detected was Asp present as a major dimethylated and a minor monomethylated derivative with an overall stoichiometry of 0.9 residue per DAF polypeptide. Since N^{α}-radiomethylation occurs only at protein N-termini (Haas & Rosenberry, 1985), this finding is indicative of N-terminal Asx, an assignment consistent with Edman sequencing results which indicate N-terminal Asp.[2] In addition to the labeled amino acids, 1.8 and 0.8 labeled ethanolamine and glucosamine, respectively, were present per DAF polypeptide, amounts comparable to those in E^{hu} AChE (Table I).

To investigate the possible location of the ethanolamine and glucosamine residues in a C-terminal glycolipid, radiomethylated E^{hu} DAF was digested with papain, an enzyme that

[2] M. E. Medof, unpublished results.

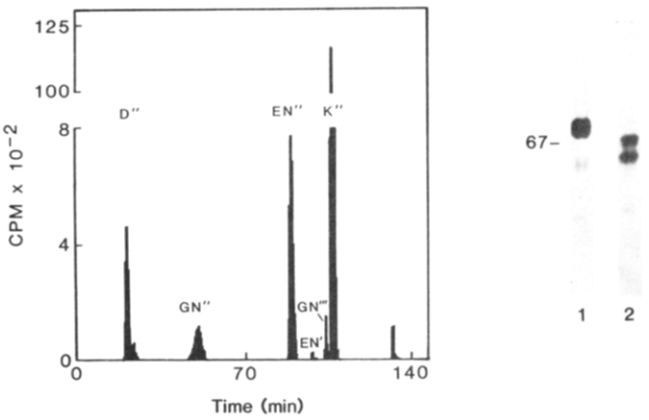

FIGURE 1: Identification of residues radiomethylated in DAF on an automated amino acid analyzer. E^{hu} DAF (0.6 μg) was radiomethylated, hydrolyzed, and fractionated as outlined under Materials and Methods. D, Asp; GN, glucosamine; EN, ethanolamine; K, Lys. One prime denotes the *N*-monomethyl and two primes the *N*,*N*-dimethyl derivatives, respectively, with Lys methylation occurring on the ε-amino group. Labeled components were identified by elution times and, in the case of N^{α},N^{α}-dimethyl- and N^{α}-methyl-Asp, by their coelution with radiomethylated standards (Haas et al., 1986; Haas & Rosenberry, 1985). The glucosamine assignment also was based on the presence of a characteristic acid hydrolysis degradation product, GN''' (Haas et al., 1986). The gel shows the fluorographic analysis following SDS gradient PAGE of ^{14}C-radiomethylated DAF (1000–2000 ^{14}C cpm). Lane 1, intact E^{hu} DAF; lane 2, E^{hu} DAF following papain digestion.

releases the C-terminal dipeptide of E^{hu} AChE with the attached glycolipid anchor (Dutta-Choudhury & Rosenberry, 1984). Following digestion, the DAF product was reisolated by gel exclusion chromatography. The estimated Stokes radius of the labeled protein in 0.1% Triton X-100 decreased from 8.2 nm to about 6 nm, a shift comparable to that observed following papain cleavage and release of E^{hu} AChE from Triton X-100 micelles (Dutta-Choudhury & Rosenberry, 1984). SDS–PAGE (Figure 1) indicated that the products consisted of two large fragments that appeared 9 and 16 kDa smaller than intact E^{hu} DAF. Amino acid analyses of these fragments demonstrated that radiomethylated Asp and Lys were quantitatively retained, while labeled ethanolamine and glucosamine had been completely removed (Table I). Assuming that papain cleavage involves only peptide bonds and that E^{hu} DAF is devoid of polypeptide cross-links, the retention of the labeled N-terminal Asp indicates that the small fragments containing the labeled ethanolamine and glucosamine were derived from the E^{hu} DAF C-terminus. This result is comparable to that obtained following papain digestion of E^{hu} AChE and confirmed by Edman sequencing of the AChE anchor fragment (Haas et al., 1986).

Release of Cell-Associated DAF by PI-PLC. A property of the C-terminal glycophospholipid anchoring structures of trypanosome mfVSGs and Thy-1 antigen is their susceptibility to cleavage by PI-PLC, an enzyme which removes diacylglycerol from PI and releases these proteins from cells as hydrophilic derivatives (Ferguson et al., 1985b; Low & Kincade, 1985). To determine if DAF is also anchored by an interaction involving PI, the susceptibility of endogenous DAF in E^{hu} to release by PI-PLC was examined.

The results are shown in Figure 2A. In multiple experiments, up to 18 ng of DAF antigen (corresponding to 15% of E^{hu}-associated DAF) was detected in the supernatant of cells treated with 10 μg/mL PI-PLC, while <7 ng was found in the supernatant of cells treated with as much as 10-fold higher concentrations of PC-specific PLC from *B. cereus*, PLC from *C. perfringens* (not shown), phospholipase A2, or trypsin (not

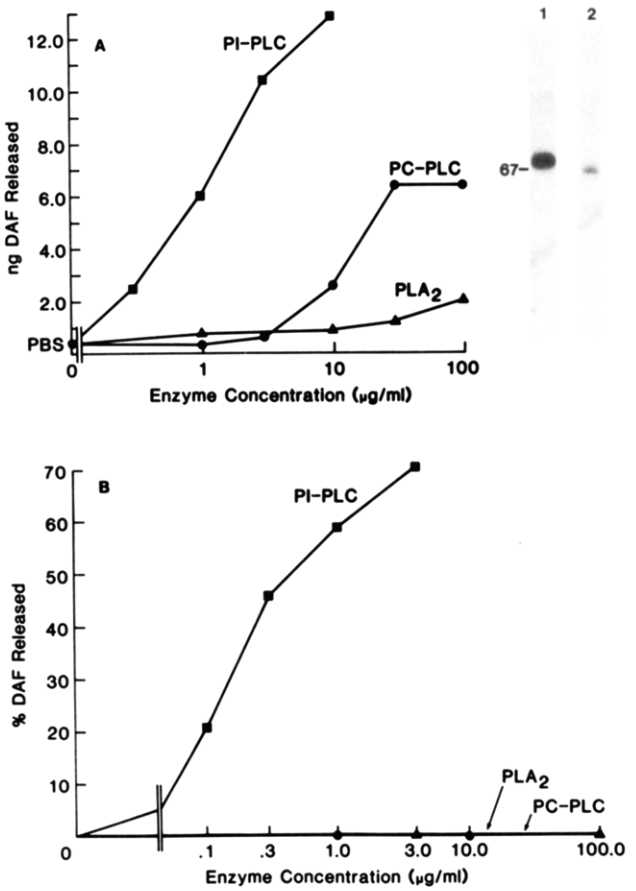

FIGURE 2: Release of DAF from E^{hu} by PI-PLC. (A) Aliquots (100 μL) of intact E^{hu} (5×10^9/mL) were incubated at 37 °C with increasing concentrations of PI-PLC (■) or, as controls, with *B. cereus* PC-PLC (●) or phospholipase A2 [PLA_2 (▲)]. After 1 h, supernatants were separated, cells washed and extracted with 1% NP40, and the supernatants and cell extracts assayed for DAF antigen by two-site radiometric assay employing monoclonal anti-DAF antibodies. (B) E^{sh} (5×10^8 mL) were preincubated at 30 °C for 2 h with E^{hu} DAF (2.5 μg/mL in GVB^{2+} containing 0.005% NP40). After being thoroughly washed, the E^{sh} bearing incorporated DAF in their membranes were incubated at 37 °C for 60 min with 0–3 μg/mL PI-PLC (■) or 1–100 μg/mL PL-PLC (●) or PLA_2 (▲), and the extent of release of DAF antigen was quantitated as in (A). The gel in (A) shows SDS–PAGE analysis of ^{125}I-labeled DAF released by PI-PLC. E^{hu} (5×10^8/mL) surface labeled with ^{125}I by Iodogen were incubated at 37 °C for 1 h with 10 μg/mL PI-PLC. Following separation of supernatants and extraction of cells with NP40, DAF in each sample was immunoprecipitated and subjected to SDS–PAGE and radioautography. Lane 1, cell extract from PI-PLC incubation; lane 2, supernatant from PI-PLC incubation.

shown). In contrast, in control studies in which the enzyme effects on red cell associated CR1 were assessed, no CR1 antigen was detected in the supernatants of PI-PLC-treated E^{hu} while >5 ng (>75%) was removed from the E^{hu} by trypsin (data not shown). Similar results were obtained if washed stroma were substituted for intact E^{hu}.

In another set of experiments, the effect of PI-PLC on DAF reconstituted into cell membranes was examined. Purified E^{hu} DAF was incorporated into E^{sh}, and after thorough washing, the DAF-containing cells were incubated with PI-PLC or control PC-PLC and the supernatants assayed for DAF antigen as above. As shown in Figure 2, panel B, ~70% of the reconstituted DAF was released by PI-PLC while <5% was released by PC-PLC.

The apparent size of the DAF species released by PI-PLC was compared to that of native E^{hu} DAF by using ^{125}I surface-labeled E^{hu}. The labeled DAF antigen released by PI-PLC treatment and residual E^{hu}-associated DAF were purified

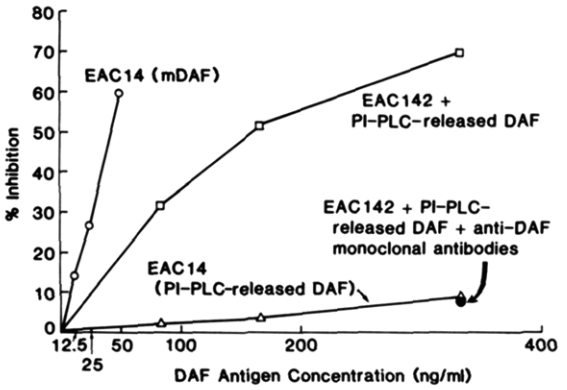

FIGURE 3: Effect on C3 convertase of DAF released from E^{hu} by PI-PLC. Increasing concentrations of native E^{hu} DAF (○) or DAF released by PI-PLC in GVB^{2+} (△) were incubated at 30 °C for 30 min with $E^{sh}AC14$. After being washed, the treated cells were incubated with excess C2 followed by C3–9, and C4b hemolytic activity was measured. Alternatively, $E^{sh}AC14$ were treated at 30 °C for 5 min with C2 (see Materials and Methods), and after being washed, the resulting EAC142 were decayed at 30 °C in the presence of DAF released by PI-PLC (□), and residual C4b2a activity was quantitated. As control (●), pooled anti-DAF monoclonal antibodies (5 μg/mL each) were added, and the experiment was repeated. Data are plotted as percent inhibition of hemolysis relative to control incubations without added DAF.

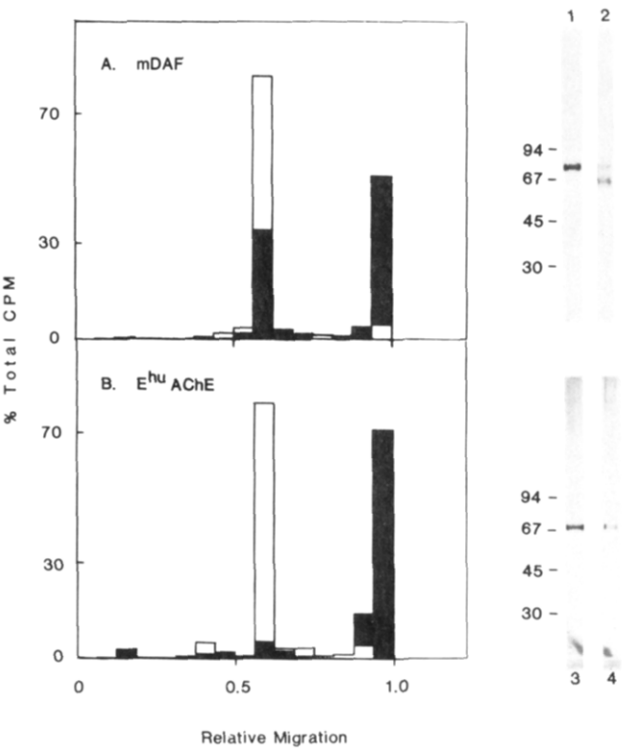

FIGURE 4: Cleavage of [^{125}I]TID-labeled anchor fragments from E^{hu} DAF and AChE by deamination. Purified solutions (500–700 μL) of E^{hu} DAF (7 μg) and AChE (6 μg) labeled with [^{125}I]TID (20000 cpm) were incubated with $NaNO_2$ at pH 4.0 for 3 h at 40 °C, neutralized, and radiomethylated as outlined under Materials and Methods. Control labeled samples without $NaNO_2$ or radiomethylation also were prepared. Aliquots (50 μL, 1000 ^{125}I cpm) were subjected to (reduced) SDS gradient PAGE followed either by silver staining or by gel slicing (1-cm segments). Profiles of ^{125}I cpm from the sliced gel lanes are shown as percent of total label recovery for (A) DAF and (B) AChE samples. Shaded histograms are the deaminated samples, and open histograms are the nondeaminated controls. Total label recoveries ranged from 70% to 80% of the input. These deaminations (performed at 40 °C) cleaved 55% of the label from DAF and 73% from AChE. The gels show the corresponding samples on parallel gel lanes stained with silver, with the controls in lanes 1 and 3 and deaminated samples in lanes 2 and 4.

by specific immunoprecipitation and analyzed by SDS–PAGE and radioautography. The released DAF antigen (Figure 2A, lane 2) appeared ~3 kDa smaller than the residual DAF in the cell extracts (lane 1). In contrast, the small amount of DAF antigen detected in the supernatants from the control buffer incubation or in incubations with PC-PLC (data not shown) appeared unchanged in size from the residual cell DAF. Western blot analysis of the supernatants from E^{sh} reconstituted with E^{hu} DAF and digested with PI-PLC yielded similar results (not shown).

The hydrophobic properties of the 67-kDa DAF form released by PI-PLC were next compared to those of native E^{hu} DAF. DAF released by PI-PLC from (unlabeled) E^{hu} was incubated with phenyl-Sepharose at 0 °C for 15 min, and the percentage of DAF antigen adsorbed to the resin was quantitated by radioimmunometric assay of the resin supernatant. In contrast to detergent-depleted native E^{hu} DAF which was >95% absorbed by the resin, PI-PLC-released DAF was <10% adsorbed (data not shown). To assess whether the DAF released by PI-PLC could membrane-incorporate, its ability to insert into $E^{sh}AC14$ and intrinsically inhibit assembly of C4b2a on the washed intermediates was evaluated (Figure 3). In contrast to the marked reduction in C4b hemolytic activity induced by native DAF, the DAF species released by PI-PLC had little or no effect on the C4b hemolytic titer of the cells. Extraction of the intermediates with NP40 and radioimmunometric assay revealed that, while cells incubated with native E^{hu} DAF contained incorporated DAF in the surface membrane, the cells incubated with DAF released by PI-PLC contained no incorporated antigen (data not shown). However, when $E^{sh}AC14$ were first treated with C2 and the resulting $E^{sh}AC142$ incubated with the PI-PLC-released DAF species, marked inhibition of C3 convertase activity was observed. The addition of monoclonal anti-DAF antibodies completely reversed the inhibition, confirming that the activity resided in the released DAF. On a weight basis, the efficiency of the inhibition was comparable to that observed following treatment of the $E^{sh}AC142$ with C4bp, which inhibits C4b2a activity by competitively displacing C2a from C4b (Nussenzweig & Melton, 1980). The DAF product released by PI-PLC thus was markedly less hydrophobic than E^{hu}DAF and unable to incorporate into cell membranes, but retained its functional site.

Cleavage of the E^{hu} DAF and AChE Anchors by Deamination. Since E^{hu} DAF and AChE contain glucosamine with a free primary amino group, the susceptibility of these glucosamine residues to nitrous acid deamination and cleavage at their glycosidic linkages (Shively & Conrad, 1976) was explored. To follow the reaction, the two proteins were radiolabeled with [^{125}I]TID, a reagent which reacts with hydrophobic components (Brunner & Semenza, 1981) and which has been shown to label selectively the E^{hu} AChE glycolipid anchor (Roberts & Rosenberry, 1986a). Deamination of the [^{125}I]TID-labeled DAF and AChE resulted in release of the label as illustrated in Figure 4. The release was more efficient with decreasing pH (from 5 to 4) and increasing temperature (from 37 to 50 °C). At pH 4.0 and 40 °C, deamination released 73% of the label from AChE and 55% from DAF, mostly within 1 h (Figure 4). At pH 4.0 and 50 °C, deamination released 86% and 70% of the label, respectively. The reaction did not significantly alter the mobility of AChE on SDS–PAGE (see Figure 4B gel) but generated a second DAF band on SDS–PAGE that appeared 9 kDa smaller than E^{hu} DAF (Figure 4A gel). The lack of a molecular weight change following cleavage of AChE is similar to that observed following cleavage of bovine erythrocyte AChE with PI-PLC.[3]

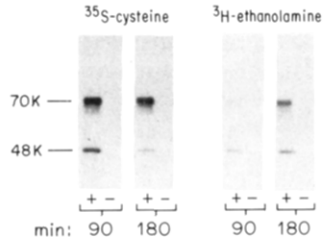

FIGURE 5: Biosynthetic incorporation of [^{35}S]Cys and [^{3}H]-ethanolamine into DAF protein in HeLa cell cultures. Four identical semiconfluent HeLa cell monolayer plates were divided into two sets and incubated with [^{35}S]Cys (50 μCi/plate) or [^{3}H]ethanolamine (66 μCi/plate) for 90 or 180 min as outlined under Materials and Methods. One plate from each set was removed at each time point, and newly synthesized DAF was immunoprecipitated and examined by SDS–PAGE followed by fluorography as outlined under Materials and Methods. Lanes designated (−) are controls using nonrelevant antimalaria monoclonals in place of anti-DAF monoclonals.

Radioautography revealed no ^{125}I label in the new band (data not shown), indicating that the label was confined to the fragment cleaved from E^{hu} DAF by deamination. These labeled DAF fragments and the corresponding fragments released from [^{125}I]TID-labeled E^{hu} AChE by deamination behaved identically in their relative elution volumes from Sephadex LH-60 in ethanol/88% formic acid and in their mobility on silica thin-layer chromatography in chloroform/methanol/water (65:25:4) (not shown).

To determine whether the ^{125}I-labeled DAF fragment released by deamination corresponded to the membrane binding domain, the mixture of residual intact and cleaved DAF from the experiment in Figure 4A was radiomethylated and applied to Sepharose CL-4B equilibrated in 20 mM sodium phosphate, pH 7. A single ^{14}C-radiomethylated peak eluted. This peak was devoid of ^{125}I label and corresponded on SDS–PAGE to the lower band (lane 2) in Figure 4A. Triton X-100 (0.1%) was introduced, and a second peak with both ^{14}C and ^{125}I label was eluted. This peak corresponded on SDS–PAGE to the upper band in the Figure 4A gel and presumably represents residual uncleaved DAF. Upon mixing with phospholipids and deoxycholate (Kim & Rosenberry, 1985), only the second peak reconstituted into liposomes (data not shown). Automated amino acid analysis revealed that both peaks contained ^{14}C-radiomethylated Lys and ethanolamine but only the second peak contained radiomethylated glucosamine. Thus, the removal of the ^{125}I-labeled fragment from E^{hu} DAF by deamination cleavage at the glucosamine residue in the anchor abolished the ability of DAF to interact with detergent micelles or liposomes.[4]

Incorporation of [^{3}H]Ethanolamine into DAF during Biosynthesis. Pulse–chase studies of DAF biosynthesis in HeLa cells using [^{35}S]Cys and [^{35}S]Met have demonstrated that the mature E^{hu} DAF molecule arises from a 48-kDa intracellular DAF precursor.[2] The marked increase in apparent size associated with conversion of the precursor into mature HeLa DAF (72 kDa) is due to addition of O-linked oligosaccharides in the Golgi (see Discussion). To determine whether ethanolamine is incorporated into DAF during de novo biosynthesis and to ascertain the intracellular site of incorporation, studies of DAF biosynthesis were performed in the presence of [^{3}H]ethanolamine. Parallel studies with [^{35}S]Cys labeling were done to facilitate the interpretation of results.

Fluorographs of newly synthesized DAF protein isolated by specific immunoprecipitation 90 and 180 min following addition of the labels are shown in Figure 5. [^{3}H]Ethanolamine-labeled bands corresponding to both the 48-kDa and the 72-kDa DAF species were observed. Similar results were obtained at 30 min (not shown). The rate of appearance of the ^{3}H-labeled 48-kDa pro-DAF band roughly coincided with that of the [^{35}S]Cys-labeled 48-kDa band, and the conversion of the ^{3}H-labeled pro-DAF to the ^{3}H-labeled 72-kDa mature E^{hu} DAF paralleled that of the ^{35}S-labeled pro-DAF. Amino acid analysis of the immunoprecipitate confirmed that ^{3}H-label incorporation into DAF during de novo biosynthesis was associated exclusively with unaltered [^{3}H]ethanolamine. Ethanolamine thus incorporates directly into DAF, and the incorporation occurs prior to processing of pro-DAF in the Golgi.

Discussion

The main finding of the present study is that DAF is anchored to E^{hu} by a C-terminal glycolipid structure similar to that contained in E^{hu} AChE (Dutta-Choudhury & Rosenberry, 1984; Haas et al., 1986; Roberts & Rosenberry, 1986a), trypanosome mfVSGs (Holder, 1983; Ferguson et al., 1985a,b), and brain and thymus Thy-1 antigen (Tse et al., 1985; Low & Kincade, 1985).

Information about this recently discovered class of anchoring structures for membrane proteins is rapidly emerging. The mfVSG structure is the best characterized so far. It consists of dimyristylphosphatidylinositol in glycosidic linkage with an oligosaccharide composed of one glucosamine, two mannose, zero to eight galactose, two phosphate, and one ethanolamine residues (Ferguson et al., 1985b). Thy-1 and E^{hu} AChE contain components consistent with a similar structure with variations. The Thy-1 structure contains primarily stearate (18:0) rather than myristate (14:0), inositol, one glucosamine, two to three mannose, zero to one galactosamine, two to three phosphate, and one to two ethanolamine residues (Tse et al., 1985). The E^{hu} AChE structure includes one saturated and one unsaturated fatty acid [primarily palmitate (16:0) and 22:4/22:5], inositol (Roberts & Rosenberry, 1985, 1986b), one glucosamine, and two to three ethanolamine residues (Haas et al., 1986). This study presents several observations which indicate that the E^{hu} DAF anchor belongs to the same class of glycolipid structures. These include the identification and localization of ethanolamine and glucosamine at or near the E^{hu} DAF C-terminus, the demonstration that E^{hu} DAF, like mfVSGs (Ferguson et al., 1985b), can be converted into a hydrophilic DAF form by reagents which cleave PI and nonacetylated glucosamine, and the discovery that HeLa cell DAF can be labeled biosynthetically with [^{3}H]ethanolamine.

One important line of evidence for the involvement of PI in the anchoring structures of several members of this family of membrane proteins has been their susceptibility to release from membranes in situ by highly purified PI-PLC from *Staphylococcus aureus* (Ferguson et al., 1985b; Low & Kincade, 1985). Early studies by Low and co-workers demonstrated that this enzyme quantitatively cleaves integral membrane AChEs from torpedo electric organ (Futerman et al., 1983) and from pig and ox erythrocytes (Low & Finean, 1977). Recent studies have shown that it efficiently releases Thy-1 antigen from murine thymocytes and EL4 thymoma cells (Low & Kincade, 1985) and rapidly releases VSGs from trypanosomes (Ferguson et al., 1985b). Experiments with [^{3}H]myristic acid labeled mfVSGs have demonstrated that PI-PLC acts within PI to release soluble VSGs (sVSGs) that

[3] W. L. Roberts, B. H. Kim, and T. L. Rosenberry, unpublished observations.

[4] Under the relatively harsh deamination conditions, AChE lost all enzymatic activity and exhibited heterogeneous aggregation on Sepharose CL-4B. Furthermore, hydrolysate analyses following deamination and radiomethylation of both AChE and DAF showed loss of ^{14}C-labeled N-terminal amino acids and modified ^{14}C-labeled ethanolamine elution even in the uncleaved pools.

retain anchor-associated oligosaccharide and inositol from *sn*-1,2-[^{3}H]dimyristylglycerol. In the present study, PI-PLC released DAF antigen from E^{hu} (Figure 2). This release was specific since it did not occur with 10-fold higher concentrations of *B. cereus* PC-PLC, *C. perfringens* PLC, phospholipase A2, or trypsin. In marked contrast, CR1 of E^{hu} was unaffected by PI-PLC but was totally cleaved by trypsin under the same conditions. The PI-PLC cleavage of E^{hu} DAF was inefficient in that only 15% of the cell-associated DAF was released (Figure 2A). This result parallels findings with E^{hu} AChE. Even though inositol has been identified in the AChE anchor structure (Roberts & Rosenberry, 1986b), PI-PLC released only ~5% of the enzyme from E^{hu} (Low & Finean, 1977) or from E^{hu} AChE-containing liposomes (Roberts & Rosenberry, 1986a). The PI-PLC stock used in this study was the same as that shown to cleave nearly 100% of mfVSGs (Ferguson et al., 1985b) and in control studies cleaved 90% of bovine erythrocyte AChE.[3] The reason for the lower efficiency of PI-PLC cleavage of DAF and AChE in E^{hu} is not yet clear. It could arise from chemical modification of core PI, from steric effects which influence the accessibility of PI-PLC to the anchors of these proteins, or from the presence of covalently linked phospholipids in addition to PI. Since only a portion of the added E^{hu} DAF was taken up into E^{sh}, our finding that incorporated DAF in E^{sh} membranes was more efficiently cleaved by PI-PLC than endogenous E^{hu} DAF does not distinguish among these possibilities.

Analysis of the structure and functional activity of the DAF derivative generated by PI-PLC cleavage revealed that E^{hu} DAF exhibits properties of an amphipathic protein similar to those of mfVSGs (Ferguson et al., 1985b; Borst & Cross, 1982) and E^{hu} AChE (Dutta-Choudhury & Rosenberry, 1984). The DAF derivative released by PI-PLC, like sVSGs and the product of papain digestion of E^{hu} AChE, was similar in size (Figure 2A) but markedly less hydrophobic than membrane DAF. The PI-PLC-generated DAF derivative did not adsorb to phenyl-Sepharose and was unable to alter C4b sites on E^{sh}AC14 cells, an activity requiring cell membrane incorporation. However, it retained the ability to accelerate decay of preformed C4b2a on E^{sh}AC142 cells with an efficiency comparable on a molar basis to that of C4bp. This observation demonstrated that the active site of DAF is distinct from its membrane anchor and like the enzymatic site of AChE (Dutta-Choudhury & Rosenberry, 1984) is located in the hydrophilic portion of the E^{hu} DAF molecule. The similarity in structure and function of the PI-PLC-released DAF species to urine DAF, in conjunction with recent findings (Medof et al., 1985a) that large amounts of membrane DAF are expressed by epithelial cells lining the urinary tract, raises the possibility that urine DAF, like sVSGs (Ferguson et al., 1985a), might arise via the action of an endogenous PLC. Structural analyses of urine DAF for components of the glycolipid anchor proximal to the site of PI-PLC cleavage should clarify this issue.

In previous studies, radiomethylation of E^{hu} AChE revealed that its glycolipid anchor contained glucosamine and ethanolamine with free primary amino groups in addition to an ethanolamine in amide linkage to the C-terminus (Haas et al., 1986). In contrast, in mfVSGs only a single ethanolamine which is in amide linkage to the C-terminus has been reported (Holder, 1983; Ferguson et al., 1985b). The close structural relationship of the glycolipid anchors of E^{hu} DAF and AChE is indicated by the similar amounts of ethanolamine and glucosamine accessible to radiomethylation (Table I) and the release of these labeled residues in small C-terminal fragments by papain digestion. Papain generated only a single fragment from E^{hu} AChE that corresponded to the C-terminal dipeptide His-Gly linked to the glycolipid (Haas et al., 1986), while the two residual DAF bands observed on SDS–PAGE following papain treatment (Figure 1B) indicated that the digestion of E^{hu} DAF was less selective. Sequencing of E^{hu} DAF C-terminal fragments will be required to establish whether the labeled ethanolamine and glucosamine in E^{hu} DAF are linked to the DAF C-terminal amino acid as in E^{hu} AChE.

To relate the glucosamine detected in DAF directly to the DAF anchor and to further examine the extent of similarity between the anchors of E^{hu} DAF and AChE, the two proteins were subjected to nitrous acid deamination (Shively & Conrad, 1976). This procedure was used to demonstrate that the glucosamine in the mfVSG anchor is in glycosidic linkage to PI (Ferguson et al., 1985b). The deamination reaction cleaved the two proteins in a parallel fashion. Cleavage of DAF correlated with loss of its ability to interact with detergent micelles or liposomes. Radiomethylation of the deaminated DAF and AChE products demonstrated that they retained ethanolamine but not glucosamine and thus established that radiomethylated ethanolamine is located between the C-terminal amino acid of each protein and the glucosamine glycosidic bond. Analysis of the released [^{125}I]TID-labeled DAF and AChE anchor fragments demonstrated that they were identical by thin-layer chromatography criteria.[5] Additional analyses to precisely identify the released fragments are in progress and will be the subject of another communication.

To further characterize the E^{hu} DAF anchor and to obtain information about its intracellular site of incorporation, studies of DAF biosynthesis were carried out by using [^{3}H]ethanolamine, an anchor-specific probe which biosynthetically incorporates into mfVSGs (Rifkin & Fairlamb, 1985) and Thy-1 antigen.[6] These studies demonstrated direct uptake of the label into DAF during de novo DAF biosynthesis. Label initially appeared in 48-kDa pro-DAF and subsequently in 72-kDa mature HeLa cell DAF. Previous studies in HL60 cells (Lublin et al., 1985) have established that the apparent size of pro-DAF is decreased by about 4 kDa upon treatment with endoglycosidase H, and studies in HeLa cells[2] have shown that pro-DAF can be labeled with [^{3}H]mannose, demonstrating that it contains high-mannose N-linked carbohydrates. The apparent molecular weight of mature DAF is decreased by about the same amount upon treatment with endoglycosidase F or during biosynthesis in the presence of tunicamycin (Lublin et al., 1985).[2] However, only the mature DAF and not pro-DAF is labeled by the biosynthetic precursor *N*-[^{3}H]acetylgalactosamine,[2] a component of *O*-linked carbohydrates. Thus, the marked increase in apparent DAF size from 48 to 72 kDa is due mainly to addition of *O*-linked sugars in the Golgi, and the appearance of [^{3}H]ethanolamine label in pro-DAF indicates that incorporation of ethanolamine into DAF glycolipid occurs prior to *O*-linked sugar addition in the Golgi.

A similar conclusion of early biosynthetic uptake of [^{3}H]-ethanolamine into trypanosome mfVSGs was reached when the ^{3}H label was shown to be detected within 1 min of the appearance of newly synthesized VSG polypeptide (Rifkin & Fairlamb, 1985). Moreover, recent studies (Ferguson et al.,

[5] Added in proof: In contrast to the [^{125}I]TID-labeled deamination fragment of bovine erythrocyte AChE, only a portion of the [^{125}I]TID deamination fragments of E^{hu} DAF and AChE migrated as [^{125}I]TID-labeled PI.

[6] S. H. Fatemi, R. Haas, T. L. Rosenberry, and A. M. Tartakoff, unpublished observations.

1986) using [^{14}C]myristate have yielded the same results and have further indicated that anchor-associated components may be added simultaneously; i.e., the glycophospholipid anchor may be preassembled and added en bloc. Sequence analyses of VSG cDNAs predict a hydrophobic C-terminal extension of 17–23 amino acids that is not present in mature mfVSG proteins (Borst & Cross, 1982), and it has been suggested that this extension peptide is cleaved and the glycophospholipid anchor substituted as an immediate posttranslational step while VSGs are still associated with the rough endoplasmic reticulum.

The realization that multiple membrane proteins are deficient in PNH cells (Medof et al., 1985c) has suggested that these proteins might share a common structural element that is missing or defective in PNH. Our demonstration that DAF and AChE are anchored to E^{hu} by apparently similar glycolipid structures indicates one such element and raises the possibility that a defect in the assembly or attachment of this glycolipid could account for the common deficits of the two proteins in PNH E^{hu}. In support of this possibility, alkaline phosphatase, another protein deficient in PNH cells (Lewis & Dacie, 1965), can be released from porcine tissue by PI-PLC (Low & Zilversmit, 1980) and thus may be anchored by the same glycolipid structure.

Acknowledgments

We thank Dr. M. Low for providing PI-specific phospholipase C, Patricia Brandt for technical assistance, and Bonnie Berry and Janice Gresh for manuscript preparation.

References

Asch, A., Kinoshita, T., & Nussenzweig, V. (1985) *J. Exp. Med. 162*, 221–226.

Borst, P., & Cross, G. A. M. (1982) *Cell (Cambridge, Mass.) 29*, 291–303.

Brunner, J., & Semenza, G. (1981) *Biochemistry 20*, 7174–7182.

Dutta-Choudhury, T. A., & Rosenberry, T. L. (1984) *J. Biol. Chem. 259*, 5653–5660.

Ferguson, M. A. J., Haldar, K., & Cross, G. A. M. (1985a) *J. Biol. Chem. 260*, 4963–4968.

Ferguson, M. A. J., Low, M. G., & Cross, G. A. M. (1985b) *J. Biol. Chem. 260*, 14547–14555.

Ferguson, M. A. J., Duszenko, M., Lamont, G. S., Overath, P., & Cross, G. A. M. (1986) *J. Biol. Chem. 261*, 356–362.

Futerman, A. H., Low, M. G., & Silman, I. (1983) *Neurosci. Lett. 40*, 85–89.

Haas, R., & Rosenberry, T. L. (1985) *Anal. Biochem. 148*, 154–162.

Haas, R., Brandt, P. T., Knight, J., & Rosenberry, T. L. (1986) *Biochemistry 25*, 3098–3105.

Herz, F., & Kaplan, E. (1973) *Pediatr. Res. 7*, 204–214.

Hoffman, E. M. (1969) *Immunochemistry 6*, 391–403.

Holder, A. A. (1983) *Biochem. J. 209*, 261–262.

Hsiung, L.-M., Barclay, A. N., Brandon, M. R., Sim, E., & Porter, R. R. (1982) *Biochem. J. 203*, 293–298.

Iida, K., Mornaghi, R., & Nussenzweig, V. (1982) *J. Exp. Med. 155*, 1427–1438.

Kerr, M. A. (1980) *Methods Enzymol. 80*, 54–64.

Kim, B. H., & Rosenberry, T. L. (1985) *Biochemistry 24*, 3586–3592.

Kinoshita, T., Medof, M. E., Silber, R. S., & Nussenzweig, V. (1985) *J. Exp. Med. 162*, 75–92.

Lewis, S. M., & Dacie, J. V. (1965) *Br. J. Haematol. 11*, 549–556.

Low, M. G., & Finean, J. B. (1977) *FEBS Lett. 82*, 143–146.

Low, M. G., & Zilversmit, D. B. (1980) *Biochemistry 19*, 3913–3918.

Low, M. G., & Kincade, P. W. (1985) *Nature (London) 318*, 62–64.

Lublin, D. M., Pangburn, M. K., Krsek-Staples, J., & Atkinson, J. P. (1985) *Complement 2*, 49.

Medof, M. E., Kinoshita, T., & Nussenzweig, V. (1984) *J. Exp. Med. 160*, 1558–1578.

Medof, M. E., Walter, E., Rutgers, J., Knowles, D., & Nussenzweig, V. (1985a) *Complement 2*, 53.

Medof, M. E., Kinoshita, T., Silber, R., & Nussenzweig, V. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2980–2984.

Medof, M. E., Nussenzweig, V., Kinoshita, T., Gottlieb, A., Hall, S., Silber, R., & Rosse, W. F. (1985c) *Complement 2*, 53.

Medof, M. E., Haas, R., Walter, E. I., & Rosenberry, T. L. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 382.

Nelson, R. A., Jensen, J., Gigli, I., & Tamura, N. (1966) *Immunochemistry 3*, 111–135.

Nicholson-Weller, A., Burge, J., Fearon, D. T., Weller, P. F., & Austen, K. F. (1982) *J. Immunol. 129*, 184–189.

Nicholson-Weller, A., March, J. P., Rosenfeld, S. I., & Austen, K. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5066–5070.

Nicholson-Weller, A., March, J. P., Rosen, C. E., Spicer, D. B., & Austen, K. F. (1985) *Blood 65*, 1237–1244.

Nussenzweig, V., & Melton, R. (1980) *Methods Enzymol. 80*, 124–133.

Pangburn, M. K., Schreiber, R. D., & Muller-Eberhard, H. J. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5430–5434.

Pangburn, M. K., Schreiber, R. D., Trombold, J. S., & Muller-Eberhard, H. J. (1983b) *J. Exp. Med. 157*, 1971–1980.

Rifkin, M. R., & Fairlamb, A. H. (1985) *Mol. Biochem. Parasitol. 15*, 245–256.

Roberts, W. L., & Rosenberry, T. L. (1985) *Biochem. Biophys. Res. Commun. 133*, 621–627.

Roberts, W. L., & Rosenberry, T. L. (1986a) *Biochemistry 25*, 3091–3098.

Roberts, W. L., & Rosenberry, T. L. (1986b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1816.

Rosenberry, T. L., & Scoggin, D. M. (1984) *J. Biol. Chem. 259*, 5643–5652.

Rosse, W. F., & Parker, C. J. (1984) *Clin. Haematol. 14*, 105–125.

Shively, J. E., & Conrad, H. E. (1976) *Biochemistry 15*, 3932–3942.

Tack, B. F., Janotova, J., Thomas, M. L., Harrison, R. A., & Hammer, C. H. (1980) *Methods Enzymol. 80*, 64–101.

Tse, A. G. D., Barclay, A. N., Watts, A., & Williams, A. F. (1985) *Science (Washington, D.C.) 230*, 1003–1008.