THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 43, Issue of October 25, pp. 40472–40478, 2002
Printed in U.S.A.

A Novel Human Erythrocyte Glycosylphosphatidylinositol (GPI)-anchored Glycoprotein ACA

ISOLATION, PURIFICATION, PRIMARY STRUCTURE DETERMINATION, AND MOLECULAR PARAMETERS OF ITS LIPID STRUCTURE*

Received for publication, March 12, 2002, and in revised form, August 2, 2002
Published, JBC Papers in Press, August 7, 2002, DOI 10.1074/jbc.M202416200

Zorica A. Becker Kojić‡ and Peter Terness

From the Institute of Immunology, Department of Transplantation Immunology, University of Heidelberg, Im Neuenheimer Feld 305, 69120 Heidelberg, Germany

A method has been elaborated to isolate and purify up to homogeneity a novel membrane glycoprotein containing a glycosyl-phosphatidylinositol (GPI) anchor by means of salting out with ammonium sulfate (40–80% saturation), followed by preparative SDS-PAGE, chromatography and acetone precipitation. The preparation obtained was homogeneous upon electrophoresis in the presence of 0.1% SDS after reduction with 2-mercaptoethanol. It is protein-soluble at its isoelectrical point (pH 5.5) with molecular mass of 65,000 daltons. The isolated protein is linked to the membrane via glycosylphosphatidylinositol susceptible to cleavage by purified phospholipase C. The hydrophobic portion of the glycolipid membrane anchor of the protein was radiolabeled with the photoactivated reagent 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine and hydrolyzed with glycosyl-phosphatidylinositol-specific phospholipase C, followed by enzymatic deacetylation of the remaining lipid. Thin-layer chromatography showed that the generated radiolabeled fragment migrates with the same mobility as that of variant surface glycoprotein (VSG), obtained in the same manner. In this study we describe a novel erythrocyte membrane GPI-linked protein with the structural feature of an anchor that, in contrast to other GPI-linked erythrocyte proteins, has a non-acetylated inositol ring and diacylglycerol rather than alkylacyl glycerol as a lipid tail of the anchor.

Many proteins of eukaryotic cells are anchored to the membrane by covalent linkage to glycosyl-phosphatidylinositol (GPI)[1] (1). Those proteins lack a transmembrane domain, have no cytoplasmic tail, and are therefore located exclusively on the extracellular side of the plasma membrane. Glycosylated phosphoinositol-anchored molecules are a structurally diverse family of biomolecules (1–3) that includes: protozoan coat components, activation antigens, complement regulatory proteins, adhesion molecules, membrane-associated enzymes, and many others glycoproteins. This type of cell membrane attachment implies that the protein moieties of these antigens are located outside the membrane, have potentially high lateral mobility, and may be released from the cell by the action of GPI-specific phospholipase C. GPI anchors may be considered as a reasonably well defined class of the structures because important elements are conserved over a wide range of phylogeny, from protozoan parasites to mammals. This anchor contains a linear core glycan sequence of ethanolamine-PO_4-6 Manα1–2Manα1–6Manα1–4GlcNH_2-α1–6*myo*-inositol-1-PO_4-lipid. The heterogeneity among different GPI-anchored proteins occurs mainly in the lipid composition. The variant surface glycoprotein (VSG) anchors contain only dimyristoyl phosphatidylinositol (4), whereas GPIs of *Leishmania* promastigote surface proteinase and many mammalian GPI-anchored proteins including those on the erythrocyte such as acetylcholinesterase (AchE), decay-accelerating factor (DAF), and membrane inhibitor of reactive lysis (MIRL) (5), contain almost exclusively 1-alkyl-2-acylinositol phospholipid, which appear to be characteristic of human anchors in general (6). In addition, some GPI anchors contain an additional fatty acid (palmitate) in a hydroxyester linkage to the inositol ring, which renders them resistant to the action of bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) and the GPI-specific phospholipase C (GPI-PLC) from *Trypanosoma brucei*. The basis of this phospholipase resistance was first described for human erythrocyte acetylcholinesterase (7), and subsequently for human erythrocyte decay-accelerating factor (5). The presence of a substitution on the 2-position of the inositol ring would explain the PI-PLC resistance of the palmitoylated anchors since the bacterial PLC enzyme operates via nucleophilic attack of the phosphorus atom by the hydroxyl group at the 2-position of the inositol ring. This finding led to the suggestion that occupation of the *myo*-inositol 2-position hydroxy group by palmitate would automatically preclude the action of this enzyme (8). As shown for DAF and some other GPI-linked proteins they can exhibit differential inositol acetylation, and this structural variation is regulated in a cell-specific fashion, but can also be protein-dependent (9). The biological relevance of GPI anchor structural variability among different blood cell types remains incompletely understood. The presence of additional fatty acid chain (acylation) in the hydrophobic domain probably improves the attachment of the molecule to the external leaflet of the mem-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence reported in this paper has been submitted to the SWISS-PROT/TrEMBL Database under the accession no. P83408.

‡ Supported by the University of Heidelberg, Hochschulsonderprogramm II (HSP II). Present address: Otto Meyerhof Centre, Dr. Terness Laboratory, Im Neuenheimer Feld 350, 69120 Heidelberg, Germany. To whom correspondence and reprint requests should be addressed. Tel.: 49-6221-380986; Fax: 49-6221-382292; E-mail: ZBecker-Kojic@t-online.de.

[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; VSG, variant surface glycoprotein; AchE, acetylcholinesterase; DAF, decay-accelerating factor; MIRL, membrane inhibitor of reactive lysis; PI-PLC, phosphatidylinositol specific phospholipase C; GPI-PLC, glycosylphosphatidylinositol specific phospholipase C; PI-PLD, phosphatidylinositol phospholipase D; CRD, cross-reacting determinant; GK, glycerokinase; LDH, lactate dehydrogenase; [^{125}I]TID, 3-(trifluoromethyl)-3-(m[^{125}I]iodophenyl) diazirine; TLC, thin-layer chromatography; s/mVSG, soluble/membrane VSG; IEF, isoelectrical focusing.

brane. The presence of this acyl-chain on the inositol ring in erythrocyte-associated anchors renders these structures resistant to cleavage by PI-PLC and less sensitive to PI-PLD cleavage when purified. The resistance to be released by phospholipases, either by blocking the lytic mechanism of a phosphatidyl-specific phospholipase C or by retaining membrane association following cleavage of phosphatidic acid by anchor-specific phospholipase D, may enhance the membrane stability of these proteins over time, consistent with the longer (120 day) life span in the circulation of erythrocytes as compared with leukocytes. We report here on a novel GPI-linked erythrocyte membrane protein with an anchor sensitive to phospholipases C and unusual structure of the lipid portion of protein, distinct from that of other erythrocyte (DAF, LFA, MIRL, and acetylcholinesterase) proteins.

EXPERIMENTAL PROCEDURES

Purification and Isolation of Protein—Human blood withdrawn from healthy donor was centrifuged at 1500 × *g* at 4 °C for 10 min. Plasma and buffy coat were removed by aspiration, and packed cells were washed twice in 150 mM/15 mM sodium phosphate (pH 7.6) and lysed in CH_3COOH/distilled water. Membranes were pelleted at 10,000 × *g* for 15 min and washed with extraction buffer, 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.25 M sucrose. This step was repeated until the ghosts showed no visual evidence of residual hemoglobin. (Three washes were usually required). After the last wash, the ghosts were resuspended in the same buffer. The pellet was frozen in fluid nitrogen for 15 min and thawed at 25 °C. This procedure was repeated three times. The homogenate was then adjusted to 400 mM with respect to KCl and centrifuged at 25,000 × *g* for 60 min. The supernatant was collected and adjusted to 70% saturation with $(NH_4)_2SO_4$ (Sigma) and stirred for 30 min. The precipitated proteins were collected by centrifugation, redissolved in the extraction buffer, and precipitated again with 40% saturation with $(NH_4)_2SO_4$ (see Fig. 2*A*). The precipitated proteins were stirred again for 30 min on ice, redissolved in extraction buffer, and dialyzed against storage buffer (40 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 1 mM EDTA, 0.25 M sucrose), and stored at −20 °C. The pure amphiphilic form of protein was obtained by further preparative steps including SDS-gel electrophoresis. The gel bands of the protein were excised and crushed in 25 mM Tris-base, 192 mM glycine, and 0.035% SDS (pH 8.3), and the supernatant was collected after overnight incubation at 4 °C. Purified protein was dialyzed against the storage buffer $(NH_4)_2CO_3$, precipitated with ice cold acetone in ethanol/dry ice bath, and stored at 4 °C. The protein concentrations were measured by the methods of Bradford (10).

Electrophoretic Methods—SDS-PAGE was performed on a linear gradient gel of 4–15% polyacrylamide in the discontinuous buffer of Laemmli (11). The samples were boiled for 2 min in the reducing sample buffer before electrophoresis. Protein bands were identified by silver staining or negative staining by $CuCl_2$. Molecular weights were estimated by comparison with the position of molecular weight standards purchased from Bio-Rad Laboratories (München, Germany). Gels were fixed with methanol/acetic acid or ethanol/acetic acid and visualized with Coomassie Blue or by standard silver staining.

Isoelectrical Focusing—A total of 1–5 ng of protein proband was subjected to PhastGel isoelectrical focusing (IEF) gradient media supplied by Amersham Biosciences. The samples are applied ~10 mm from a cathode. Additional controls were done applying samples next to an anode and a cathode. The bands were visualized using the silver staining method according to manufacturer's instruction. For standards, the Amersham Biosciences Broad pI Calibration KIT on PhastGel IEF 3–9 was used.

Chemical Deglycosylation—Chemical deglycosylation was performed to prepare ACA protein for primary structural analysis. A protein sample free of salts, metal ions, and detergents was treated with anhydrous trifluoromethane sulfonic acid (Oxford GlycoScience, UK) for 4.5 h in a freezer, neutralized, and recovered in 0.5% ammonium bicarbonate. The neutralized reaction mixture was then dialyzed against the same buffer, and precipitated deglycosylated protein isolated directly by centrifugation.

Protein Sequence Analysis—Chemically deglycosylated protein was treated with trypsin or Asp-N at 37 °C for 18 h. Peptides were separated by C_{18} reverse phase-high pressure liquid chromatography. N-terminal sequences were determined by automated Edman degradation using gas phase sequencer. Subsequently, a search in the human sequence data bank was performed (www.ncbi.nlm.nih.gov./entrez/query.Fcgi?db = protein).

Cell-free Extracts—Preparation of ghosts was done according to protocol approved by the Institutional Review Board of the New York Blood Center. Briefly, about 2.3×10^6/ml packed red cells and erythroleukemia cells (K-562) were washed once with phosphate-buffered saline buffer at 5000 rpm for 5 min. After the supernatant was decanted, the cells were lysed with 1:20.5 dilution of phosphate-buffered saline in distilled water and centrifuge at 15,000 rpm for 10 min. Supernatant was decanted, and the discrete pellet of the white cells adhering to the centrifuge tube was removed, too (12). Resuspended ghosts were washed with 10 mM Tris-HCl (pH 7.8) until they became white and fluffy. Equal volume of simple buffer was added and boiled for 2 min before subjected to SDS-PAGE using 4.5 stacking gel and 4–15% separating gel.

Immunoblotting—Membrane proteins (10–30 μg) prepared from the erythrocyte ghosts and homogenized tissue of erythroleukemia (K-652) cells were mixed with reducing sample buffer and subjected to SDS-PAGE and then transferred to nitrocellulose membrane. The filters were blocked with 3% bovine serum albumin in phosphate-buffered saline and used for testing antibody reactivity. Polyclonal mouse serum against a 65-kDa GPI-protein was used at a dilution of 1:5000. Visualization was performed using anti-IgG secondary antibody and conjugated with alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as a chromogenic substrate for alkaline phosphatase (Promega).

Digestions with Phospholipases—Digestion with glycosyl phosphatidylinositol-phospholipase C (GPI-PLC) from *Trypanosoma brucei* (Oxford GlycoScience) was performed for 1 h at 30 °C. Samples of proteins were further buffered by addition of TBS (Tris-buffered saline, 20 mM Tris-HCl, 0.15 M NaCl, containing 0.1% of Triton X-114, 10 mM 1-chloro-3-tosylamido-7-amino-2-heptanone, and 5 mM EDTA) and incubated with 5 μl (25 units/ml) of enzyme. Incubation with 5 milliunits of PI-PLC (*Bacillus cereus*, Roche Molecular Biochemicals) were performed in triethanolamine buffer (50 mM triethanolamine, 10 mM EDTA, and 10 mM sodium azide, pH 7.5) for 1 h at 37 °C. Digestion with anchor-specific phospholipase D (from bovine serum EC 3.1.4.50 Roche Molecular Biochemicals) was performed in 20 mM Tris-HCl, pH 7.4, 0.1 mM $CaCl_2$, Triton X-100 0.008% (w/vol.) for 60 min at 37 °C. After the reactions were finished, 10 μl of reducing sample buffer was added. Tubes containing the reaction mixtures were placed in a boiling water bath for 5 min, and aliquots of the samples were subsequently subjected to analysis by SDS-PAGE and silver staining (PhastGel System from Amersham Biosciences).

Anti-Cross-reacting Determinant (CRD) Assay—The GPI-PLC-, PI-PLC-, and GPI-PLD-cleaved proteins were subjected to SDS-PAGE and transferred onto nitrocellulose. After blocking, the antigens were visualized with affinity-purified anti-CRD antibody (GlycoScience, Oxford). After washing, bound anti-CRD was detected by incubation with biotinylated donkey anti-rabbit IgG and visualized with streptavidin horseradish peroxidase. As positive and negative controls, the soluble form of variant surface glycoprotein and the membrane form of ACA were blotted simultaneously and revealed with anti-CRD antibody.

Identification of Products of Phospholipases Action—Purified ACA protein was digested with PI- and GPI-phospholipases C and subjected to further hydrolysis with triacylglycerol acyl hydrolase (300 units) (*Rhizopus arrhizus* EC 3.1.1.1, Roche Molecular Biochemicals), followed by carboxylic ester hydrolase (30 units) (from pig liver EC 3.1.1.1., Roche Molecular Biochemicals) in reaction buffer containing 0.1 mg of SDS/ml for 25 min at 25 °C. Glycerol is determined as described by purchaser (Roche Molecular Biochemicals) with glycerokinase (GK) pyruvate kinase (PK), and lactate dehydrogenase (LDH) as auxiliary and indicatory enzymes (Roche Molecular Biochemicals). Lipase, esterase, PK, LDH, and GK were free from hexokinase, as well as other interfering kinases and phosphatases; ATP (Roche Molecular Biochemicals) was substantially free from ADP and P-enolpyruvate (Roche Molecular Biochemicals) from pyruvate. The decrease in the extinction at 340, 334, and 365 nm due to oxidation of NADH was measured (Fig. 1).

Radiolabeling—Purified proteins were labeled with 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine) ([^{125}I]TID) (Amersham Biosciences) by photolysis at 350 nm for 15 min as described by Roberts and Rosenberry (13). A 10-μl aliquot of [^{125}I]TID in ethanol (5–200 μCi) was added to 500 μl of buffered red blood cells ACA protein in a 1 cm^2-rubber-stoppered borosilicate glass test tube and agitated gently to ensure complete mixing. Photolysis was performed for 20 min in a Beckman spectrophotometer with the slits removed.

Thin-layer Chromatography (TLC)—Samples of GPI-PLC and PI-

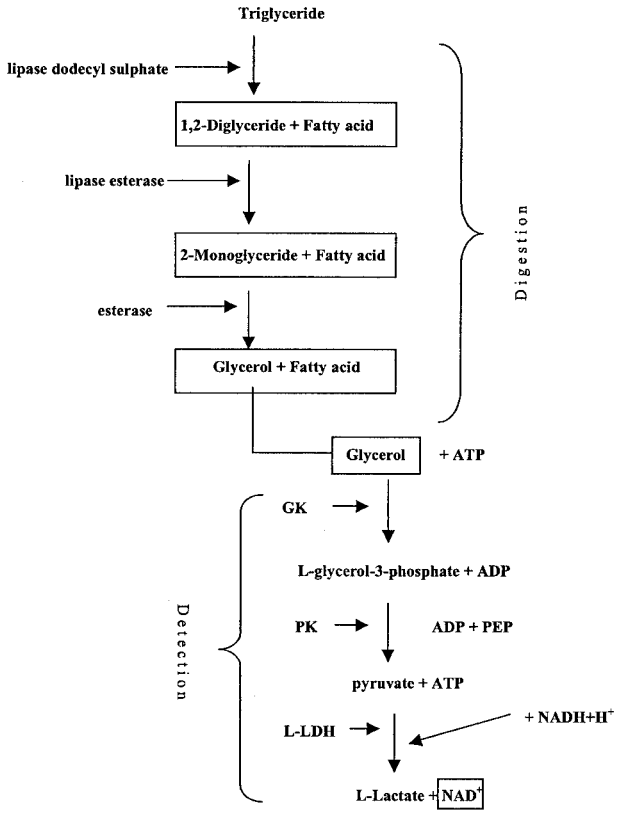

FIG. 1. **Identification of products of phospholipases action.** Enzymatic reaction leading to the total hydrolysis of lipid tail of glycosylphosphatidyl inositol of ACA and enzymatic reactions for determinations of glycerol concentration.

PLC cleaved [^{125}I]-TID-labeled proteins were subjected to further hydrolysis with triacyl glycero-protein acylhydrolase followed by carboxylic-ester hydrolase, and the lipid products of this reaction were extracted with chloroform/methanol/HCl (22:50:1) and centrifuged for 15 min at 300 × *g*. The lower phase was removed and evaporated to dryness under nitrogen. The residues were resolved in 0.1 ml of chloroform/methanol (2:1), and these solutions were quantitatively applied to TLC plates. Thin-layer chromatography was performed on 10 × 20-cm Silica Gel plates (Merck, Darmstadt, Germany) without activation. Development was either with solvent B (hexane/diethylether/acetic acid (60:30:1)) or solvent A (hexane/2-propanol, (96:4)). The positions of radioactive components were identified by autoradiography with Kodak XAR-5 film. The positions of standards were located by exposure of dried plates to iodine vapor.

Production of Polyclonal Mouse Antiserum against 65-kDa GPI-Protein—Homogeneously purified 65-kDa GPI-protein in complete Freund's adjuvant was used for immunization of 6 BALB/c mice. After 4 weeks the mice were boosted, and a week later the mice were testbled. The mice with the highest titers of anti-ACA activity in enzyme-linked immunosorbent assay screening assays were used for antiserum production and later on, after additional boosting, for the production of monoclonal antibodies.

RESULTS

Purification of ACA—A two-step purification procedure was applied for the preparation of the protein. The first step includes the disruption of the hemoglobin-free membrane followed by repeated precipitation with ammonium sulfate of various saturation grades and preparative electrophoresis (Fig. 2*A*). The purity of preparations was evaluated by SDS-PAGE (Fig. 2, *A* and *B*) and IEF, (Fig. 3, *A* and *B*). Preparations were obtained from various batches of pooled human erythrocytes. No degradation products were identified in any preparation.

Amino Acid Sequencing of Purified ACA—Purified and chemically deglycosylated ACA was subjected to N-terminal amino acid sequencing. No sequence was obtained, indicating a blocked N terminus. Therefore, the protein was inactivated and trypsinized, and sequence was established for 11 internal peptides (Fig. 4). Only peptide A shows homology with the 1230–1246 region of the α-chain of human erythrocyte spectrin (14).

Digestion with Phospholipases—We further focused our attention on the biochemical characterization of this protein, particularly of its lipid portion, and compared it with some other GPI-linked molecules expressed on the surface of human erythrocytes.

The susceptibility of a protein to release from a membrane surface by bacterial PI-PLC is the original indicator for the

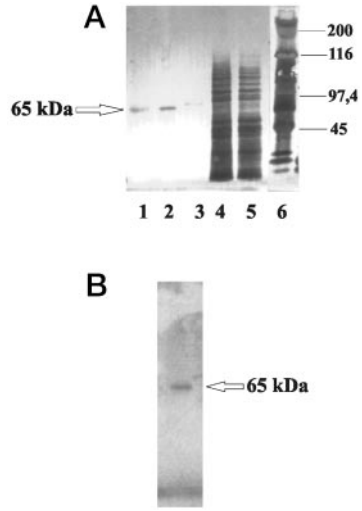

FIG. 2. **Purification and isolation of ACA.** Electrophoresis of erythrocyte ghost ammonium sulfate protein fraction and purified ACA was performed on 4–15% SDS-PAGE. *A*, *lanes 1*, *2*, *3*, purified ACA protein obtained from various batches of erythrocytes; *lanes 4* and *5*, 40% ammonium sulfate-precipitated protein extracts obtained from various batches of erythrocyte membrane; and *lane 6*, molecular mass marker. *B*, purified ACA protein, molecular mass 65 kDa.

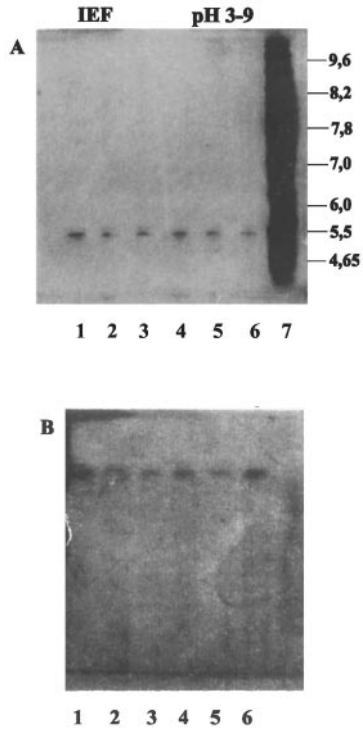

FIG. 3. **Isoelectrical focusing of ACA.** Electrophoresis of purified ACA on IEF-gradient gel was performed. *A*, *lanes 1–6*, purified ACA obtained from various batches of erythrocytes starting from anode; *B*, starting from cathode. *Lane 7* in *A*, IEF broad pI calibration standards.

Peptide A: D-L-V-P-L-E-D-K-V-T-I-L-G-M-T-A

Peptide B: K-L-A-L-S-A-D-D-P-G-F-H-N-F-S-H-Q-R-Q-T

Peptide C: D-Q-Q-T-T-S-H-S-S

Peptide D: V-L-E-I-M-L-P

Peptide E: F-Q-D-E-S-E-A-N-K

Peptide F: M-K-Y-V-N-F-K-F-Y-F

Peptide G: N-L-D-F-M-T-W-G-V-T-K-V-T-Y-I-G-Q-P-T-G-G

Peptide H: L-L-M-D-N-N-E-A-V-H

Peptide I: F-D-Q-A-W-A-D-T-A-H-T-W

Peptide J: K-L-D-D-I-Q-K-D-M-Y-S-Q-Q-D-T

Peptide K: G-V-W-I-M-K-N-Q-I-T

FIG. 4. **Sequence of tryptic peptides of ACA.**

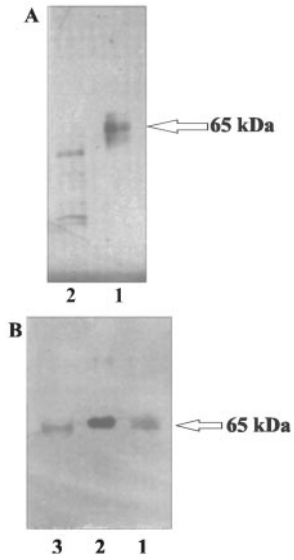

FIG. 5. **SDS-PAGE analysis of phospholipase-digested proteins.** Cleavage of ACA with GPI-PLC, PI-PLC, and GPI-PLD. *A*, *lane 1*, purified ACA; *lane 2*, PI-PLC-treated ACA. *B*, *lane 1*, purified ACA; *lane 2*, GPI-PLD-treated ACA; and *lane 3*, ACA treated with GPI-PLC.

presence of a GPI anchor. However, some GPI anchors are resistant to hydrolysis by bacterial PI-PLC, such as erythrocyte membrane proteins, and this resistance depends on additional acylations on the inositol ring (15). To investigate whether this protein is anchored in the membrane bilayer by covalent attachment of the C-terminal amino acid residue to a glycosylphosphatidylinositol, we studied the specific hydrolysis of the phosphodiester bond of phosphatidylinositol with different anchor-degrading enzymes. We first observed that the effect of PI-PLC on the erythrocyte membrane protein fraction led to a disappearance of a very faint electrophoretical band, which was later identified as our new human erythrocyte protein. Because of the very low abundance of this protein on the surface of erythrocytes, we further characterized the lipid portion of this molecule on the purified protein. Phospholipases C hydrolyzes the phosphodiester linkage to produce 1,2-diacylglycerol, alkylacylglycerol and the phosphorylated polar head group, while the phospholipases D hydrolyzes the phosphodiester linkage to produce a phosphorylated 1,2-diacylglycerol or alkylacylglycerol and the polar head group with an exposed hydroxyl group. As shown in Fig. 5*A*, there was no portion of the ACA protein that remained resistant to anchor-degrading phospholipases. The native, intact protein exhibits slow migration, indicative of detergent micellar association. The PI and GPI-PLC hydrolyzed protein of red blood cells ACA exhibited more rapid migration, characteristic of a detergent-free hydrophilic species.

The ACA protein treated with glycosyl-phosphatidylinositol phospholipase D shows no changes in electrophoretic mobility when compared with the native protein (Fig. 5*B*). The lower electrophoretic mobility of GPI-PLD-digested protein, when compared with the PI-PLC digested one, is due to the lack of a phosphate (negative charge) on the inositol ring of the degraded protein.

Presence of the CRD Epitope on ACA—A polyclonal antibody raised to soluble VSG cross-reacts with other unrelated GPI-anchored proteins. This CRD is only exposed when the protein is converted into a hydrophilic form by the action of either bacterial PI-PLC or eukaryotic GPI-PLC. In the case of the soluble form of variant surface glycoprotein, three overlapping epitopes are involved in this recognition: the inositol 1,2-cyclic phosphate generated on phospholipase C cleavage of the anchor, the non-acetylated glucosamine residue, and the variable galactose branch. It is also known that regarding mammalian proteins the major epitope involved in this recognition is the inositol 1,2-cyclic phosphate (16). Thus, the recognition of a protein following phospholipase C cleavage by an anti-CRD antibody is the most powerful evidence for the presence of a GPI anchor. In the next set of experiments we sought to determine whether anti-CRD antibodies recognized a soluble form of isolated ACA protein. As demonstrate here, human erythrocytes ACA exhibit >99% PI-PLC sensitivity to the action of phospholipases C. Glycosyl-phosphatidylinositol phospholipase C-digested protein was electrophoresed and then subjected to Western blotting using anti-CRD antibody (Fig. 6). As positive control, the membrane form of variant surface glycoprotein (mVSG) was digested with the same anchor-degrading phospholipases. As negative control, the native form of ACA and GPI-PLD-cleaved protein were blotted simultaneously and labeled with anti-CRD antibody. The GPI-PLD-cleaved molecules do not react with anti-CRD antibodies indicating that this enzyme leaves phosphodiacylglycerol and 1,6-*myo*-inositol as reaction products, destroying the CRD antigenic determinant by preventing the formation of D-myo-inositol 1,2-cyclic phosphate. The membrane form of ACA was also used as a negative control; as expected, it was not recognized by anti-CRD antibodies.

Identification of the Lipid Substituents of the ACA Anchor—PI- and GPI-PLC-digested purified protein was further hydrolyzed with triacylglycerol acyl-hydrolyze, giving the 1,2-diglyceride and fatty acid as first reaction products followed by carboxylic ester hydrolase, an enzyme that accelerates the total hydrolysis by a specific cleavage of soluble glyceride, leaving glycerol and fatty acids as the final reaction products (17). Glycerol was then determined enzymatically with GK, PK, and LDH as auxiliary and indicatory enzymes. The scheme of all enzymatic reactions leading to total hydrolysis of the lipid tail of the ACA anchor and enzymatic reactions for determinations of glycerol are outlined in Fig. 1. The decrease in extinction at 340, 334, and 365 nm due to oxidation of NADH is measured, and released glycerol calculated in g/liter. The amounts of released glycerol by hydrolysis with PI and GPI-phosphatidylinositol-specific phospholipases followed by specific deacetylation were similar, indicating the same specific activity of both enzymes on ACA. (Table I). To further investigate the structure of the hydrophobic domain in red blood cells ACA, particularly the fatty acids composition, we analyzed the fragments gener-

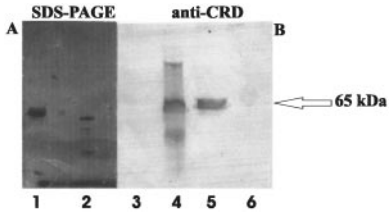

FIG. 6. **Cross-reacting determinant epitope on ACA.** CRD is generated by the action of GPI-PLC. *A*, SDS-PAGE; *lane 1*, purified native ACA protein; *lane 2*, purified ACA GPI-PLC cleaved. *B*, immunoblotting with anti-CRD antibody; *lane 3*, mf ACA (native protein); *lane 4*, GPI-PLC digested form of variant surface glycoprotein; *lane 5*, ACA digested with GPI-PLC; and *lane 6*, ACA digested with GPI-PLD.

TABLE I
Determination of glycerol concentration

Data show the amount of released glycerol after total hydrolysis of *ACA* lipid tail.

	Release of glycerol	
	PI-PLC	*GPI-PLC*
65 kDa	0.045 g/liter	0.038 g/liter

ated by total hydrolysis of the anchor. We used the photoactivated reagent [^{125}I]TID, which was shown to partition strongly in favor of the lipid phase of membranes. The photogenerated carbene labels the lipid groups of intrinsic membrane proteins in a highly selective manner. Samples of [^{125}I]TID-labeled, purified proteins were subjected to hydrolysis with GPI-phospholipase C. The lipid products of these reactions were further hydrolyzed with highly specific lipases, triacylglycerol acylhydrolase, and carboxylic-ester hydrolyze as described under "Experimental Procedures." The released radiolabeled fragments were extracted and analyzed by thin layer chromatography and autoradiography. The membrane form of variant surface glycoprotein from *T. brucei* was [^{125}I]TID-labeled and hydrolyzed in the same manner as ACA and subjected to thin-layer chromatography as a control (Fig. 7).

The major lipid species obtained by total hydrolysis of anchor of ACA exhibited the same mobility as labeled fatty acid tail of commercially available VSG, indicating the same 1,2-diacyl myristate structure. This structure differs from other erythrocyte GPI-linked proteins, which have alkyl-acyl glycerol and different fatty acids as a lipid tail of a molecule.

Generation of an ACA-specific Antibody and Detection of Its Expression on Erythrocytes—We established a purification method to obtain large amounts of protein necessary for immunization of 6 BALB/c mice according to standard procedures. To improve our understanding of this novel molecule, we first produced a mouse polyclonal antibody and tested its specificity with different samples of crude protein extract, purified protein, red cell membrane, and cell-free extracts. The specificity of the polyclonal antibody was tested in Western blots with various protein fractions. Ammonium sulfate protein extract obtained from erythrocyte membranes as described under "Experimental Procedures" was dissolved in storage buffer dialyzed against the same buffer and subjected to reducing SDS-PAGE. The homogeneously purified ACA protein and protein extracts obtained from erythrocyte ghosts and ghosts obtained from human erythroleukemia cell line (K-562) were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The polyclonal mouse serum specifically reacted with the 65-kDa band corresponding to ACA protein (Fig. 8). No reaction with other proteins was observed. The faint band of erythrocyte crude protein extract shows a high specificity of antibody to this novel antigen and a very low abundance of this molecule in the plasma membrane of red blood cells compared with human blood malignant cells.

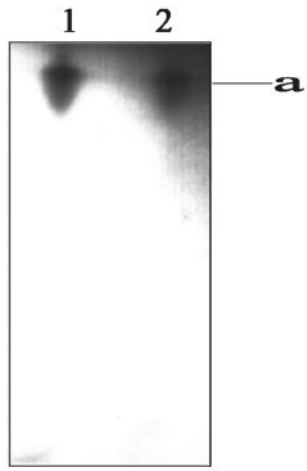

FIG. 7. **Silica TLC analysis of fragments generated by hydrolysis of anchors.** Samples of [^{125}I]TID-labeled, purified proteins were hydrolyzed with GPI-PLC. The lipid products of this reaction were further hydrolyzed with highly specific lipases. Radiolabeled fragments were extracted and analyzed by TLC. Myristic acid was used as standard. *Lane 1*, commercially available mVSG used as control was labeled, digested with GPI-PLC and further hydrolyzed as described for ACA; and *lane 2*, ACA protein GPI digested and further hydrolyzed.

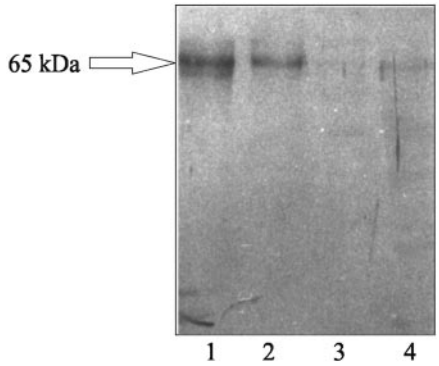

FIG. 8. **Western blotting with anti-ACA polyclonal antibody.** Proteins were subjected to SDS-PAGE under reducing conditions, transferred to nitrocellulose, and revealed with mouse antibody to ACA. *Lane 1*, crude protein extract obtained from erythrocyte membrane; *lane 2*, purified erythrocyte ACA protein; *lane 3*, erythrocyte ghosts; and *lane 4*, ghosts prepared from erythroleukemia cells.

DISCUSSION

We describe here the isolation and purification up to homogeneity of a novel human erythrocyte membrane protein called ACA. The protein was identified first, on SDS-PAGE as a faint band of ~65 kDa in a protein fraction obtained from erythrocyte ghosts. After the protein extract was treated with PI-PLC the faint band disappeared, indicating the existence of an anchor on this protein. For purification of this protein of unknown function, a classical protein chemical method was established. For further characterization of this protein, we provide two lines of evidence. The first line of evidence indicates the homogeneous protein preparation with an isoelectrical point at pH 5.5 and molecular mass of 65,000 Daltons. Mouse polyclonal antiserum raised against the purified protein specifically recognizes a protein with the same molecular mass in various protein and cell-free extracts. The second line of evidence indicates that erythrocyte ACA anchor structure differs from that of other GPI-linked erythrocyte membrane proteins. The ability of bacterial PI-PLC to release proteins attached to the membrane has permitted the identification of several phosphatidylinositol-anchored proteins (18). PI-PLC and GPI-PLC treatment of ACA extracted from red blood cells results in a

shift in the electrophoretical gel indicating a phosphoinositol structure on this protein. As judged from the gel, the whole amount of the protein was hydrolyzed, indicating that the anchor of the protein exists on the surface of the erythrocyte membrane exclusively in a non-acetylated form, which means that the *myo*-inositol 2-position hydroxy group is not additionally acetylated in ACA molecule. Additional evidence supporting this lipid structure of ACA was provided by immunoblotting with anti-CRD antibody including appropriate controls. In immunoblot analysis, purified anti-CRD antibody recognizes the phospholipase-solubilized form of ACA. The protein was recognized by anti-CRD antibody after PI-PLC digestion because this antibody reacts to a large extent with the inositol 1,2-cyclic phosphate epitope generated after PI-PLC cleavage. The treatment with GPI-PLD, however, results in the complete loss of this epitope due to inositol-1,2-cyclic-phosphate removal. Phospholipase D degraded a lipid anchor other than phospholipase C by leaving phospholipid as final reaction product. Furthermore, the anti-CRD antibody also failed to recognize the membrane form of ACA protein, indicating the specificity of the reaction. Specific hydrolysis of the lipid tail of the anchor provides evidence for the diacyl nature of glycerol binding. The first enzyme, triacylglycerol-acylhydrolase from *Rhizopus arrhizus*, belongs to that group of esterases that hydrolyze substrates quantitatively to glycerol and fatty acids, especially the α and α'-esters of glycerol and long chain fatty acids. Carboxylic ester hydrolysis, with its high specificity to monoglycerides, was facilitating the total hydrolysis of the anchor. Glycerol was a final product of these reactions, and its amount was determined according to standard procedure (17). The amounts of glycerol released from ACA by enzymatic deacetylation with bacterial and eukaryotic phospholipases was similar in both cases, but bacterial PI-phospholipase C seemed to be more active than eukaryotic GPI-phospholipase. In this paper, we documented that [^{125}I]TID specifically labels the hydrophobic domain of ACA. After total lipid hydrolysis, extraction of fatty acids, and analysis on silica thin-layer chromatography exclusively myristic acid was found. All these data provide a strong evidence that ACA protein has an anchor based on diacylmyristate, a structure very similar to that of mVSG of *T. brucei.* Previous characterizations of different GPI-anchored proteins on the surface of erythrocytes such as DAF, CD59, LFA-3, and AchE, have shown that these proteins exhibit differential inositol acylation, a structural variation regulated in a cell-specific fashion (5). It seems that the precursor pools for both PLC-sensitive and PLC-resistant anchors exist in a number of cells and that the proportion of attached glycolipid anchors arising from these two pools is both tissue and protein specific (7, 9). It has recently been shown (9) that inositol acylation in mature surface proteins is regulated via posttransfer deacylation, occurring in endoplasmic reticulum immediately after GPI transfer, which in general is cell-specific but also can be protein-dependent. The structural feature of phospholipids of all these erythrocyte GPI-linked proteins is based on 1-alkyl,2-acyl-glycerol in contrast to unacetylated dimyristoylphosphatidylinositol, which is uniformly present in trypanosome membrane from variant surface glycoproteins. This first comparison of GPI anchors from different proteins expressed in the same tissue suggests that human reticulocytes produce only one type of anchor structure (19), and furthermore also GPI-anchored proteins from other cell types seem to have exclusively alkylacylglycerol as a lipid backbone (20). The experiments described in this study provide evidence that ACAs GPI anchor phospholipid head group differs from that of known erythrocytes GPI-linked proteins like DAF, acetylcholinesterase, and MIRL. The anchor we describe here consists principally of the minimum core glycan sequence Manα1–2-Manα1–6-Manα1–4-GlcN-linked to a phosphatidyl-moiety with the structure sn-1-O-acyl-2-O-acylglycerol-3-phospho-*myo*-inositol. This structure is essentially identical to dimyristoylphosphatidylinositol, which is uniformly present in *Trypanosoma* mVSG, the first protein in which GPI anchors were chemically characterized (21). Western blot analysis of whole protein extract, purified ACA, and ghosts prepared from red blood cells and cells from erythroleukemia (K-562) with polyclonal anti-mouse antibody confirm the specificity of antibody. Moreover the same analysis indicates a very low abundance of this molecule in the membrane of red blood cells and apparently much higher expression in malignant cells.

Sequence analysis of 11 peptides obtained from ACA by trypsin digestion showed no homology with protein sequences of the data bank except for peptide A (Fig. 4), which exhibited partial identity with the 1230–1246 region of human erythrocyte spectrin. Search in the human sequence data bank was not successful; therefore, we concluded that the ACA sequence is not contained in the Human Genome data base.

We report here for the first time the existence of a novel erythrocyte GPI-phospholipase C-sensitive GPI-linked protein with the diacyl structure of the lipid backbone. The functional relevance of this molecule should be further elucidated. In fact, very little is known about the functional significance of these variations in the fatty acid linkage and composition of glycosylphosphatidyl inositol among various cell types. They are certainly not relevant for their anchoring function but may profoundly influence their sensitivity to endogenous phospholipases C or D as well as the biological activity of the glycerolipid derivatives resulting from these hydrolysis reactions. The inositol glycan, diacylglycerol, and phosphatidic acid internally generated are well known intermediates in membrane signaling events and activate the cascade of intracellular second messengers (22). Diacylglycerol and phosphatidic acid are all mitogenic (23). Diacylglycerol is involved in protein kinase C activation (24), and phosphatidic acid can induce growth factor-like effects (25), including expression of tyrosine kinases and stimulation of DNA synthesis. The objective of our future work will be to investigate the significance of this unusual PI structure and its consequences for the function of ACA.

Acknowledgment—We thank Dr. Stefan Rose-John for critical reading of the manuscript.

REFERENCES

1. Cross, G. A. M. (1990) *Ann. Rev. Cell Biol.* **6,** 1–39
2. Englund, P. T. (1993) *Ann. Rev. Biochem.* **62,** 121–138
3. Ferguson, M. A. J. (1999) *J. Cell Sci.* **112,** 2799–2806
4. McConville, M. J., and Ferguson, M. A. J. (1993) *Biochem. J.* **294,** 305–324
5. Walter, E. I., Roberts W. L., Rosenberry, T. L., Ratnoff, W. D., and Medof, M. E. (1990) *J. Immunol.* **144,** 1030–1036
6. Ratnoff, W. D., Knez, J. J., Prince, G. M., Okada, H., Lachman, P. J., and Medof, M. E. (1992) *Clin. Exp. Immunol.* **87,** 415–421
7. Roberts, W. L., Myher, J. J., Kuksis, A., Low, M. G., and Rosenberry, T. L. (1988) *J. Biol. Chem.* **263,** 18766–18775
8. Ferguson, M. J. (1992) *Biochem. J.* **284,** 297–300
9. Chen, R., Walter, E. I. Parker, G., Lapurga, J. P., Millan, J. L., Ikehara, Y., Udenfried, S., and Medof, M. E. (1998) *Proc*, *Natl. Acad. Sci. U. S. A.* **95,** 9512–9517
10. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
11. Laemmli, U. K. (1970) *Nature* **227,** 680–685
12. Dodge, J. T., Mitchell C., and Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100,** 119–130
13. Roberts, W. L., and Rosenberry, T. L. (1986) *Biochemistry* **25,** 3091–3098
14. Sahr, K E., Laurila, P., Kotula, L., Scarpa, A. L., Coupal, E., Leto, T. L., Linnenbach, A. J., Winkelmann, J. C., Speicher, D. W., Marchesi, V. T., Curtis, P. J., and Forget, B. G. (1990) *J. Biol. Chem.* **256,** 4434–4443
15. Low, M. G., Stiernberg, J., Waneck, G. L., Flavell, R. A., and Kinkade, P. W. (1988) *J. Immunol. Methods* **113**, 101–111
16. Cardoso de Almeida, M. L., and Turner, M. L. (1983) *Nature* **302,** 349–352
17. Wieland, O. H. (1984) in *Methods of Enzymatic Analysis*, (Bergmeyer, H. U., ed) 3rd Ed., Vol. VI, pp. 504–510, Verlag Chemie, Deerfield Beach, FL
18. Toutant, J. P., Richards, M. K., Drall, J. A., and Rosenberry, T. L. (1990) *Eur. J. Biochem.* **187,** 31–38

19. Rudd, P. M., Morgan, B. P., Wormald, M. R., Harvey, D. J., van den Berg, C. W., Davies, S. J., Ferguson, M. A. J., and Dwek, R. A. (1997) *J. Biol. Chem.* **272,** 7229–7244
20. Singh, N., Liang, L., Tykocinski, M., and Tartakoff, A. (1996) *J. Biol. Chem.* **271,** 12879–12884
21. Ferguson, M. A. J., and Cross, G. A. M. (1984) *J. Biol. Chem.* **259,** 3011–3015
22. Nosjean O., Briolay A., and Roux, B. (1997) *Biochim. Biophys. Acta* **1331,** 153–186
23. Fukami, K., and Takenawa, T. (1992) *J. Biol. Chem.* **264,** 10988–10993
24. Nishizuka, Y. (1984) *Nature* **308,** 693–698
25. Moolenar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J., and de Laat, S. W. (1986) *Nature* **323,** 171–173

A Novel Human Erythrocyte Glycosylphosphatidylinositol (GPI)-anchored Glycoprotein ACA: ISOLATION, PURIFICATION, PRIMARY STRUCTURE DETERMINATION, AND MOLECULAR PARAMETERS OF ITS LIPID STRUCTURE

Zorica A. Becker Kojic and Peter Terness

J. Biol. Chem. 2002, 277:40472-40478.
doi: 10.1074/jbc.M202416200 originally published online August 7, 2002