THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 267, No. 8, Issue of March 15, pp. 5569-5574, 1992
Printed in U.S.A.

Mammalian Red Cell Membrane Rh Polypeptides Are Selectively Palmitoylated Subunits of a Macromolecular Complex*

(Received for publication, May 21, 1991)

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Incubation of [^{3}H]palmitic acid, ATP, and CoA with inside-out membrane vesicles prepared from human or other mammalian red cells resulted in nearly exclusive ^{3}H-palmitoylation of the M_r = 32,000 Rh polypeptides. [^{3}H]Palmitic, [^{3}H]myristic, and [^{3}H]oleic acids were comparably esterified onto Rh polypeptides in inside-out membrane vesicles in the presence of ATP and CoA, although [^{3}H]palmitic acid was preferentially incorporated by intact human red cells. Experiments using sulfhydryl reagents or tryptic digestions suggested that multiple sulfhydryl groups on the Rh polypeptides located near the cytoplasmic leaflet of the lipid bilayer were ^{3}H-palmitoylated; the exofacial sulfhydryl group essential for Rh antigenic reactivity was not ^{3}H-palmitoylated. Transfer of fatty acid from [^{14}C]palmitoyl-CoA to sites on the Rh polypeptides occurred even after previous incubation of inside-out membrane vesicles at 95 °C or after solubilization of inside-out membrane vesicles in Triton X-100. Hydrodynamic analyses of Triton X-100-solubilized membranes surprisingly demonstrated that ^{3}H-palmitoylated Rh polypeptides behaved as a protein of apparent M_r = 170,000. These *in vitro* studies suggest that palmitoylation of Rh polypeptides occurs within a macromolecular complex by a highly selective but possibly nonenzymatic mechanism.

Although the clinically important Rh blood group antigens are of functional significance to membrane physiology, detailed molecular information remains limited. The rare Rh_{null} phenotypes lack all Rh antigens and bear incompletely defined defects in membrane lipid organization (reviewed by Agre and Cartron, 1991). Rh antigenic reactivity is known to involve phospholipid and an exofacial sulfhydryl group, but the physical size of the native Rh antigens has not been established (reviewed by Gahmberg, 1988).

The "Rh polypeptides" are a family of M_r = 32,000 red cell membrane proteins that are core structural components of the Rh antigens; however, Rh polypeptides isolated from the membrane no longer react with Rh-defining antibodies (Moore *et al.*, 1982; Gahmberg, 1982). The cDNA for one species of Rh polypeptide was recently isolated, and the derived amino acid sequence predicts a 417-amino acid integral membrane protein with 13 bilayer spanning domains; no homologies with known proteins were identified (Cherif-Zahar *et al.*, 1990; Avent *et al.*, 1990).

* This work was supported by United States Public Health Service Grants HL33991 and AA09012, and North Atlantic Treaty Organization Research Grant 0556/88 (to P. A.). 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.

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Rh polypeptides are major palmitoylated components of human red cell membranes (deVetten and Agre, 1988), and palmitoylation of cellular proteins is recognized to be a specific posttranslational modification of functional importance (reviewed by Schulz *et al.*, 1988; Towler *et al.*, 1988; Schmidt, 1989; James and Olson, 1990). The identity of the structural acylation sites, the fatty acyl selectivity, and the metabolic, evolutionary, and functional significance of Rh polypeptide fatty acylation remain unknown. This study reports several key observations of Rh palmitoylation that further explain the biochemistry of this modification.

EXPERIMENTAL PROCEDURES

Materials—[9,10^3-H]Palmitic acid, [9,10^3-H]myristic acid, [9,10^3-H]oleic acid, and [1-^{14}C]palmitoyl-CoA were from Dupont-New England Nuclear; carrier free $Na^{125}I$ was from Amersham Corp. *N*-Tosyl-L-lysine chloromethyl ketone-trypsin (250 units/mg) was from Cooper Biomedical. PMSF,[1] DTT, EDTA, EGTA, *N*-tosyl-L-lysine chloromethyl ketone, leupeptin, ATP, coenzyme A, protein molecular weight standards, Triton X-100, NEM, DTNB, palmitic acid, myristic acid, oleic acid, phospholipids, and fatty acid free bovine serum albumin were all from Sigma. Electrophoresis and immunoblot supplies were from Bio-Rad. Chloramine T, XOmat AR autoradiographic film, and developing reagents were from Kodak. Silica gel high performance thin layer chromatography plates were from Merck. Biosafe II and Biosafe NA scintillation fluids were from Research Products International Corp. Other reagents were from J. T. Baker Chemical Co.

Methods—SDS-PAGE slabs were prepared from 14% (w/v) acrylamide using the buffer system of Laemmli (1970); samples were incubated in 40 mM DTT and 1% (w/v) SDS for 10 min at 60 °C prior to electrophoresis. Membrane samples in all lanes correspond to approximately 20 μl of packed red cells except where noted. Samples containing Triton X-100 were incubated in 5-fold higher SDS concentrations. Gels were stained with Coomassie R-250, soaked for fluorographic analysis in 2,5-diphenyloxazole/dimethyl sulfoxide (22.4%, w/v) (Laskey and Mills, 1975), and exposed to film up to 2 weeks.

Preparation of Red Cell Membranes—Blood from healthy human volunteers was stored up to 2 weeks at 0 °C in acid citrate dextrose. Nonhuman blood from the Johns Hopkins University Comparative Medicine Department was stored similarly. Red cell membranes and inside-out membrane vesicles were prepared with 1 mM Na-EDTA and 0.2 mM PMSF in all buffers (Bennett, 1983).

[^{3}H]Palmitic Acid Labeling—The method was adapted from that of Staufenbiel (1988). Red cells were washed free of other elements with 0.15 M NaCl, 10 mM Na_2HPO_4, pH 7.4, and 1 mM Na-EDTA. Packed red cells (100 μl) were suspended in 5 ml of the above buffer containing 250 μCi of [^{3}H]palmitic acid (30–40 Ci/mmol) and were shaken for 15 h at 37 °C. The cells were then washed free of unadsorbed palmitic acid with chilled buffer, and membranes were prepared. Inside-out membrane vesicles equivalent to 100 μl of red cells

[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

were incubated 15 h at 37 °C in 5 ml of 10 mM Na_2HPO_4, pH 7.4, 1 mM $MgCl_2$, 1 mM Na-EGTA, 1 mM DTT, 1 μg/ml leupeptin, 0.1 mM *N*-tosyl-L-lysine chloromethyl ketone, 1 mM ATP, and 0.2 mM CoA with 250 μCi of [^{3}H]palmitic acid (or 1 μCi of [^{14}C]palmitoyl-CoA, 40–60 mCi/mmol) and were processed similarly.

Immunoblot—Proteins were electrophoretically transferred onto nitrocellulose sheets as described by Towbin *et al.* (1979) employing the buffers, wash conditions, and ^{125}I-labeled protein A described by Davis and Bennett (1982). Rabbit polyclonal antiserum to denatured Rh polypeptide (gift of K. Suyama and J. Goldstein, New York Blood Center) was employed at a dilution of 1:1000.

Sedimentation Coefficient—Inside-out membrane vesicles (100 μl) were labeled by incubation with [^{3}H]palmitic acid (above). The vesicles were then washed in 20 mM Tris-HCl, pH 7.0. Rh polypeptides were solubilized in 200 μl 3% (v/v) Triton X-100 in 20 mM Tris-HCl, pH 7.0, 1 mM Na-EDTA, 1 mM DTT, 5% sucrose (w/v), and 0.2 mM PMSF in H_2O by shaking for 60 min at 22 °C, followed by centrifugation for 30 min at 40,000 × *g* at 4 °C. Aliquots of 100 μl were loaded onto 4 ml of linear gradients of 5–20% sucrose (w/v) in the same buffer with 1% (v/v) Triton X-100, followed by sedimentation for 16 h at 167,000 × *g* at 4 °C. Protein standards (50–100 μg) were analyzed identically in separate gradients (Fig. 6*A*). After centrifugation, 100-μl fractions were analyzed for protein mobilities by Coomassie staining or fluorography of SDS-PAGE slabs. Detergent binding to the Rh polypeptides was determined using D_2O with calculations of Sadler (1979).

Stokes Radius—Inside-out membrane vesicles were labeled by incubation with [^{3}H]palmitic acid. The vesicles were then washed and solubilized as described above except that the solubilization buffer contained 100 mM NaCl and 1 mM NaN_3 but no sucrose. A 500-μl aliquot was loaded onto a 1 × 30-cm Superose 6 column running at 30 ml/h with the same buffer containing 1% (v/v) Triton X-100 while 0.5-ml fractions were collected. The column was calibrated with protein standards in the same buffer (Fig. 6*B*). Elution volumes were determined (above).

Gas-Liquid Chromatography—Rh polypeptides were isolated from ^{3}H-palmitoylated membrane vesicles by preparative SDS-PAGE. Thioesterified fatty acids were released from the protein with methanolic KOH (Schmidt *et al.*, 1979), and fatty acid methyl esters prepared with methanolic sulfuric acid were analyzed with a Hewlett-Packard 5890A gas chromatograph (Hresko *et al.*, 1990), catching exhaust vapor in chilled scintillation vials (Krakow *et al.*, 1986).

RESULTS AND DISCUSSION

Palmitoylation of the Rh Polypeptides—The M_r = 32,000 Rh polypeptides and three other membrane proteins became strongly labeled after intact human red cells were incubated with [^{3}H]palmitic acid (Fig. 1*A*, *lane 1*). These palmitoylations were previously shown to be reversible and probably represent continuous deacylation and reacylation of this set of membrane proteins (Staufenbiel, 1988; deVetten and Agre, 1988). When added directly to inside-out membrane vesicles (Steck and Kant, 1974), negligible palmitoylation of membrane proteins occurred with [^{3}H]palmitic acid alone or in the presence of either ATP or CoA (Fig. 1*A*, *lanes 2–4*). Abundant and nearly exclusive palmitoylation occurred on the Rh polypeptides when both ATP and CoA were added (Fig. 1*A*, *lane 5*). Although human and nonhuman Rh homologs were shown by iodopeptide maps to have diverged significantly (Saboori *et al.*, 1989), addition of [^{3}H]palmitic acid plus ATP and CoA to inside-out membrane vesicles prepared from red cells of five other mammalian species showed that all contained major ^{3}H-palmitoylated membrane proteins of approximately M_r = 32,000 (Fig. 1*B*).

It was hypothesized that the palmitoylation sites on the Rh polypeptides are cysteines located near the cytoplasmic leaflet of the membrane bilayer. No direct molecular analyses have yet been reported, but *in vitro* labeling studies demonstrated that ^{3}H-palmitoylation of the Rh polypeptides was reversed with 1 M hydroxylamine at pH 7 (deVetten and Agre, 1988). This treatment is known to release palmitic acid linked to cysteine residues in proteins by thioester bonds but not fatty

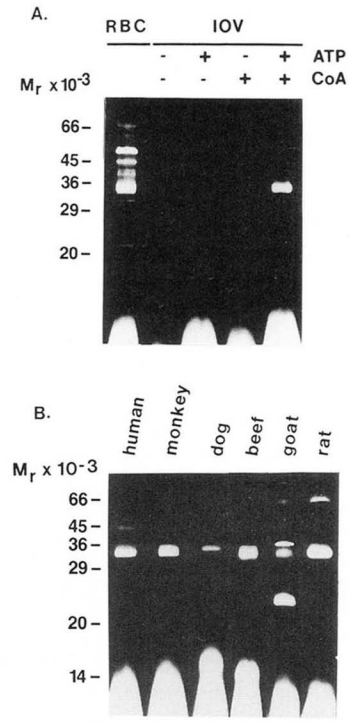

FIG. 1. **^{3}H-Palmitoylation of membranes and inside-out membrane vesicles from red cells of humans and other species.** *Panel A*, human red cells were incubated with [^{3}H]palmitic acid. Inside-out human red cell membrane vesicles were incubated with [^{3}H]palmitic acid plus 1 mM ATP, 0.2 mM CoA, or both. *Panel B*, inside-out nonhuman red cell membrane vesicles were incubated with [^{3}H]palmitic acid plus 1 mM ATP and 0.2 mM CoA. Following 15-h incubations at 37 °C, the red cell membranes and vesicles were analyzed by SDS-PAGE fluorography (see "Experimental Procedures").

acids linked through oxyester or amide bonds (Magee *et al.*, 1984). Thioether bonds are still more stable and require Raney nickel for release (Epstein *et al.*, 1990). The amino acid sequence deduced from an Rh cDNA contains 5 putative cytoplasmic cysteines and 1 exofacial cysteine (Cherif-Zahar *et al.*, 1990; Avent *et al.*, 1990). The cytoplasmic cysteines are candidate palmitoylation sites, and the exofacial cysteine may correspond to the exofacial free sulfhydryl which may be blocked with DTNB and is critical to Rh antigenic reactivity (Green, 1967, 1983).

The cytoplasmic palmitoylation site hypothesis was tested with selective sulfhydryl oxidations. NEM is known to penetrate the lipid bilayer reacting with sulfhydryls on both sides; at low concentrations, DTNB reacts primarily with local sulfhydryl groups (Smith and Ellman, 1973). Intact red cells or inside-out membrane vesicles were preincubated with 0–1.5 mM NEM or DTNB and thereafter incubated with [^{3}H]palmitic acid (red cells) or [^{3}H]palmitic acid plus ATP and CoA (inside-out vesicles). NEM blocked ^{3}H-palmitoylation in both experiments (Fig. 2, *A* and *B*); DTNB impaired ^{3}H-palmitoylation only when added to inside-out membrane vesicles (Fig. 2*B*).

Fatty Acyl Selectivity—[^{3}H]Palmitic acid was abundantly incorporated into membrane proteins in intact red cells, but [^{3}H]myristic and [^{3}H]oleic acids were incorporated negligibly (Fig. 3, *left panel*). This selectivity was lost when [^{3}H]fatty acids plus ATP and CoA were added directly to the cytoplasmic leaflet of the lipid bilayer, since [^{3}H]palmitic and [^{3}H]oleic acids were comparably incorporated into the Rh polypeptides in inside-out membrane vesicles, and [^{3}H]myristic

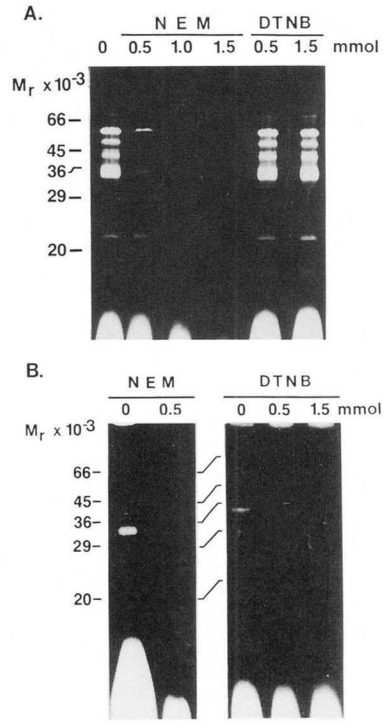

FIG. 2. **Selective inhibition of ^{3}H-palmitoylation by prior exposure of red cell membranes to sulfhydryl oxidants.** *Panel A*, human red cells were exposed for 30 min at room temperature to 0–1.5 mM NEM or DTNB, washed free of unbound reagent, and incubated with [^{3}H]palmitic acid. *Panel B*, inside-out human red cell membrane vesicles were similarly exposed to NEM or DTNB, washed, and incubated with [^{3}H]palmitic acid plus ATP and CoA. Following 15-h incubations at 37 °C, the red cell membranes and vesicles were analyzed by SDS-PAGE fluorography (see "Experimental Procedures").

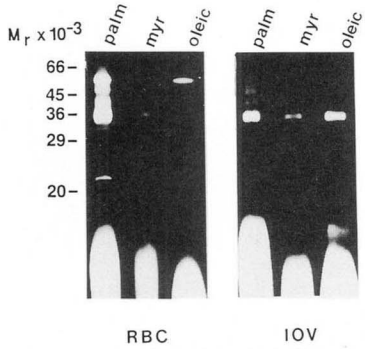

FIG. 3. **Comparison of [^{3}H]palmitic, [^{3}H]myristic, and [^{3}H] oleic fatty acylations.** *Left panel*, human red cells (*RBC*) were incubated with [^{3}H]palmitic acid, [^{3}H]myristic acid, or [^{3}H]oleic acid (10 μCi/nmol). *Right panel*, inside-out human red cell membrane vesicles (*IOV*) were similarly incubated with these [^{3}H]fatty acids plus ATP and CoA. Following 15-h incubations at 37 °C, the red cell membranes and vesicles were analyzed by SDS-PAGE fluorography (see "Experimental Procedures"). The fluorograph of the red cells (*left panel*) was deliberately overexposed to demonstrate slight fatty acylation with [^{3}H]myristic acid and [^{3}H]oleic acids.

acid was incorporated almost half as much (Fig. 3, *right panel*).

Measurement of the translocation of free [^{3}H]palmitic, [^{3}H] oleic, and [^{3}H]myristic acids across the membrane lipid bilayer was undertaken by incubating intact red cells with the [^{3}H]fatty acids followed by extraction with fatty acid-free albumin. Free [^{3}H]fatty acids were extracted from the exofacial leaflets of the membranes of intact red cells or from the cytoplasmic leaflets of inside-out membrane vesicles derived from the red cells (Table I). Free [^{3}H]palmitic acid was readily extracted from either the exofacial or cytoplasmic leaflets, while [^{3}H]palmitic acid incorporated into phospholipid was extracted from neither leaflet (Table I). When compared to [^{3}H]palmitic acid, notably less [^{3}H]myristic acid and [^{3}H]oleic acid were retained in the membranes of intact red cells, and these free fatty acids were only extractable from the exofacial leaflet (Table I).

Gas chromatography of fatty acid methyl esters eluted from the Rh polypeptides of ^{3}H-palmitoylated red cells showed that >99% of the recovered isotope cochromatographed with methylpalmitate, suggesting that the [^{3}H]palmitic acid was not transformed within the red cell membrane into another fatty acyl species. Attempts to establish the stoichiometry of Rh palmitoylation by gas chromatography were unsuccessful because of the high background contributed by fatty acids in the detergents needed for isolation of the Rh polypeptides.

Structure of Rh Palmitoylation Sites—Addition of [^{14}C] palmitoyl-CoA directly to inside-out membrane vesicles resulted in nearly exclusive labeling of the Rh polypeptides similar to that which occurred with [^{3}H]palmitic acid plus ATP and CoA (Fig. 4*A*, *lanes 1*). Prior solubilization of the vesicles in the nonionic detergent Triton X-100 reduced by >99% the ability of the Rh polypeptides to become palmitoylated with free [^{3}H]palmitic acid plus ATP and CoA but did not affect palmitoylation with preformed [^{14}C]palmitoyl-CoA (Fig. 4*A*, *lanes 2*); the latter reagent also labeled a protein of M_r = 40,000–50,000. When inside-out membrane vesicles were solubilized in denatured form in SDS, no palmitoylation of the Rh polypeptides occurred with either [^{3}H]palmitic acid plus ATP and CoA or with [^{14}C]palmitoyl-CoA (Fig. 4*A*, *lanes 3*).

Inside-out membrane vesicles were incubated for 15 min at various temperatures between 37 and 95 °C prior to incubation with [^{3}H]palmitic acid plus ATP and CoA or [^{14}C]palmitoyl-CoA at 37 °C (Fig. 4*B*, *left* and *right panels*). Palmitoylation of the Rh polypeptides with [^{3}H]palmitic acid plus ATP

TABLE I

Membrane translocation of [^{3}H]palmitic, [^{3}H]myristic, and [^{3}H]oleic acids measured by back-extraction (cpm × 10^7)

Fatty acid	Initial	Membrane associated[a]	Remaining after back-extraction[b]			
			Intact red cells		Inside-out membranes	
			Total	Phospholipid	Total	Phospholipid
Palmitic	17.4	10.2	3.71	3.41	3.53	3.38
Myristic	16.0	2.05	0.34	0.31	1.96	0.31
Oleic	16.3	4.92	1.23	1.21	4.83	0.98

[a] Intact human red cells were incubated 15 h at 37 °C with [^{3}H] fatty acids (10 μCi/nmol). The red cells were washed, and cell membrane retained radioactivity was measured (see "Experimental Procedures").

[b] The distribution of [^{3}H]fatty acids was measured by methods derived from Martin and Pagano (1987) and Bütikofer *et al.* (1990). Human red cells were incubated with [^{3}H]fatty acids as described above and divided into aliquots. A 0.1-ml aliquot of intact red cells was incubated for 15 min at 22 °C in 10 ml of 0.15 M NaCl, 7.5 mM NaH_2PO_4, 1 mM Na-EDTA, pH 7.0, with 3 mg/ml fatty acid-free bovine serum albumin; this was repeated twice. The cells were pelleted by centrifugation, and total cell membrane radioactivity was determined. Inside-out membrane vesicles were prepared from the second aliquot of red cells, and these vesicles were back-extracted as described above but without 0.15 M NaCl. Control experiments showed that <10% of radioactivity was back-extracted when albumin was omitted. Lipids were extracted from membranes in chloroform/methanol and phospholipids were isolated by thin layer chromatography (Staufenbiel, 1988) using the buffer of Schmidt *et al.* (1979).

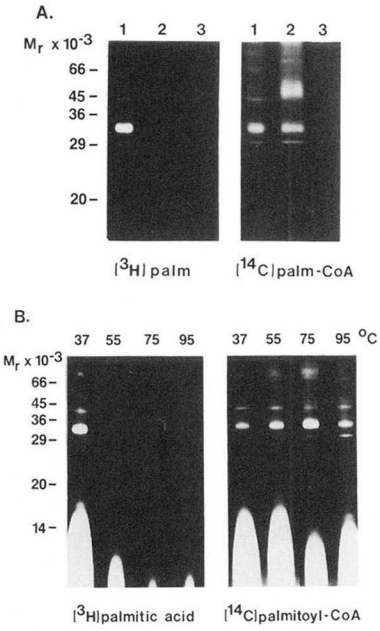

FIG. 4. **Requirements for palmitoylation of Rh polypeptides with [^{3}H]palmitic acid plus ATP and CoA or [^{14}C]palmitoyl-CoA.** *Panel A*, inside-out human red cell membrane vesicles were incubated with [^{3}H]palmitic acid plus ATP and CoA or [^{14}C]palmitoyl-CoA (*lanes 1*). Inside-out human red cell membrane vesicles were first solubilized in 3% (v/v) Triton X-100 (*lanes 2*) or 2% (w/v) SDS (*lanes 3*) and similarly incubated. *Panel B*, inside-out human red cell membrane vesicles were first incubated for 15 min at 37, 55, 75, or 95 °C, and then cooled to 37 °C and incubated with [^{3}H]palmitic acid plus ATP and CoA or [^{14}C]palmitoyl-CoA. Following 15-h incubations at 37 °C, the membrane vesicles were analyzed by SDS-PAGE fluorography (see "Experimental Procedures").

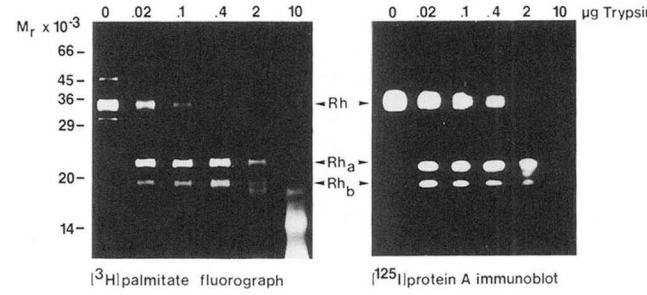

FIG. 5. **Trypsin digestion of ^{3}H-palmitoylated-Rh polypeptides within membranes.** Human Rh(D) negative red cells were obtained, and inside-out red cell membrane vesicles were prepared. The vesicles (600 µg protein/ml) were first incubated for 15 h at 37 °C with [^{3}H]palmitic acid plus ATP and CoA. The vesicles were then incubated for 1 h at 37 °C in 10 mM Na_2HPO_4, pH 7.0, 1 mM DTT, and 1 mM NaN_3 with 0, 0.02, 0.1, 0.4, 2, or 10 µg of *N*-tosyl-L-lysine chloromethyl ketone-trypsin and then heated for 60 °C for 10 min with 5 mM PMSF. Approximately 15 µg of protein was electrophoresed into SDS-PAGE slabs and visualized by fluorography (*left panel*) or analyzed by immunoblot with a polyclonal antiserum against the denatured Rh polypeptide (*right panel*, see "Experimental Procedures"). Degradation products of the Rh polypeptide are identified as Rh_a and Rh_b.

and CoA was nearly totally abolished after 55 °C preincubations, whereas transfer of palmitic acid to the Rh polypeptides from preformed [^{14}C]palmitoyl-CoA proceeded without impairment even after preincubations at 95 °C. Resistance to thermal denaturation suggests that the transfer of [^{14}C]palmitate from [^{14}C]palmitoyl-CoA is nonenzymatic, since nonenzymatic palmitoylation of myelin proteolipid protein (Bizzozero *et al.*, 1987) and rhodopsin (O'Brien *et al.*, 1987) have been described. Nevertheless, the existence of a highly stable Rh polypeptide-specific fatty acyltransferase cannot be excluded.

The hypothesis that multiple palmitoylation sites exist on the Rh polypeptides was investigated by proteolytic degradation of the ^{3}H-palmitoylated Rh polypeptides. Inside-out membrane vesicles were incubated with [^{3}H]palmitic acid plus ATP and CoA prior to digestion with various concentrations of trypsin followed by SDS-PAGE and fluorography. The ^{3}H-palmitoylated M_r = 32,000 Rh polypeptide disappeared, and two ^{3}H-palmitoylated fragments, Rh_a, M_r = 21,000, and Rh_b, M_r = 19,000, were generated which remained stable over nearly a 100-fold range of trypsin concentrations (Fig. 5, *left panel*). These fragments correspond to the two Rh polypeptide fragments observed on immunoblots stained with a polyclonal antibody raised to denatured Rh polypeptides (Fig. 5*B*). Densitometric analysis of the fluorograph demonstrated that the sum of the radiointensities of the two fragments equaled that of the original Rh polypeptide.

Palmitoylation of the Rh polypeptides appears to occur on either side of the site at which trypsin has been shown to cleave the Rh polypeptides into NH_2- and COOH-terminal fragments (Suyama and Goldstein, 1990, 1992). The amino acid sequence deduced from the Rh cDNA predicts a trypsin digestion site on a cytoplasmic loop (residues 188–205) which falls between the 5 predicted cytoplasmic cysteines occurring in sequence repeats, (11)Cys-Leu-Pro, (185)Cys-Leu-Pro, and (310)Cys-Leu-Pro-Val-Cys-Cys (Cherif-Zahar *et al.*, 1990; Avent *et al.*, 1990). Attempts to isolate the ^{3}H-palmitoylated peptides derived from proteolytically digested Rh polypeptides for chemical analyses have not yet succeeded because we have been unable to elute the exceedingly hydrophobic fragments from reverse-phase chromatography columns.

The Rh Cys-Leu-Pro motif might provide a free sulfhydryl group within a hydrophobic region located adjacent to the cytoplasmic leaflet of the lipid bilayer. No highly conserved sequence motif surrounding palmitoylation sites has been reported for several known palmitoylated proteins. Nevertheless, the fatty acylation sites established within other palmitoylated proteins are cysteines located near the cytoplasmic leaflet of the lipid bilayer and flanked by charged residues, hydrophobic residues, and glycine or proline (reviewed by Schmidt, 1989). The sequences surrounding the palmitoylation site established in the red cell band 3 protein (Okubo *et al.*, 1991) and the predicted palmitoylation sites in the Rh polypeptide share these features.

Physical Behavior of the Palmitoyl-Rh Polypeptide Membrane Complex—The size of the native Rh membrane complex has not been established (reviewed by Gahmberg, 1988). The mobility of ^{3}H-palmitoylated Rh polypeptides solubilized in Triton X-100 was determined by velocity sedimentation through sucrose gradients. The ^{3}H-palmitoylated Rh polypeptides migrated with a sedimentation coefficient = 8.6 S; this peak ran far below the detergent-phospholipid-fatty acid micelles which remained near the top of the gradients (Fig. 6*A*). Analyses of dog and rat membrane vesicles revealed ^{3}H-palmitoylated structures of a similar size (data not shown). Sedimentation analyses were repeated in buffers made from D_2O, yielding a sedimentation coefficient = 6.3 S. This density shift permitted calculation of partial specific volume, v = 0.817 ml/g, and estimation of detergent-bound, 0.667 mg of Triton X-100/mg of protein (Table II). The ^{3}H-palmitoylated Rh polypeptides were also analyzed by gel filtration in 1% (v/v) Triton X-100 (Fig. 6*B*). The Rh polypeptides migrated as a single peak with apparent Stokes radius = 52.4 Å, a value corresponding to the apparent radius of the detergent-Rh

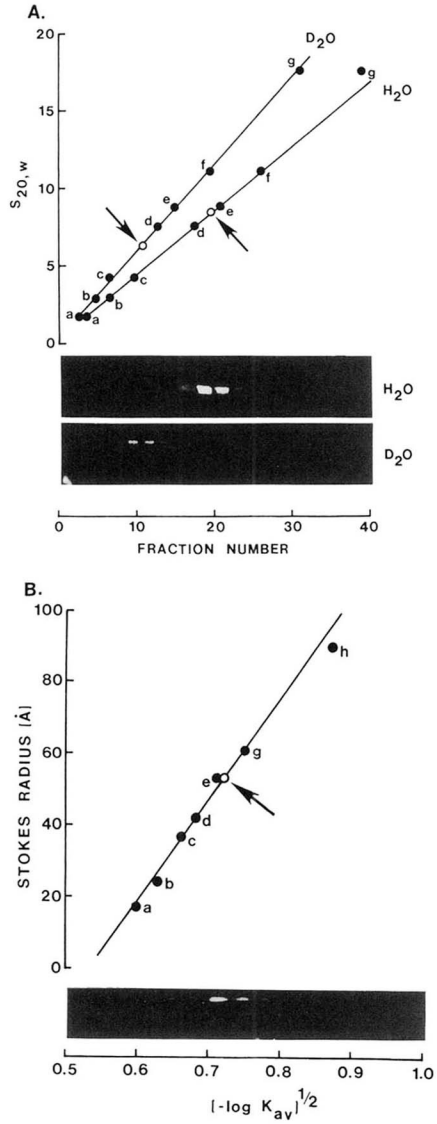

FIG. 6. **Determination of the physical size of the Rh polypeptide complex.** *Panel A*, determination of the sedimentation coefficient by ultracentrifugation. Inside-out human red cell membrane vesicles were incubated for 15 h at 37 °C with [^{3}H]palmitic acid plus ATP and CoA, solubilized in 3% Triton X-100, and analyzed by sedimentation through 4-ml linear gradients of 5–20% sucrose (w/v) containing 1% (v/v) Triton X-100 (see "Experimental Procedures"). Mobility of the ^{3}H-palmitoylated-Rh polypeptides was assessed by SDS-PAGE fluorography (*insets*). The following protein standards with known sedimentation coefficients and Stokes radii were analyzed identically in separate gradients. *a*, cytochrome *c*: 17 Å, 1.8 S; *b*, carbonic anhydrase: 24 Å, 2.7 S; *c*, bovine serum albumin: 37 Å, 4.3 S; *d*, alcohol dehydrogenase: 42 Å, 7.6 S; *e*, β-amylase: 53 Å, 8.9 S; *f*, catalase: 11.2 S; *g*, ferritin: 61 Å, 17.6 S; and *h*, thyroglobulin; 85 Å, 19.4 S. Similar sedimentations were performed in D_2O to correct for detergent binding to proteins. *Panel B*, determination of Stokes radius by nondenaturing gel filtration. Inside-out human red cell membrane vesicles were incubated with [^{3}H]palmitic acid plus ATP and CoA, solubilized as described in *panel A*, and analyzed by filtration through a Superose 6 column (see "Experimental Procedures").

protein complex and is likely to be somewhat larger than the actual Stokes radius of the protein (le Maire *et al.*, 1989). The existence of large, monodisperse peaks during sedimentation (Fig. 6*A*) and gel filtration studies (Fig. 6*B*) using a 0.1–1.0% range of Triton X-100 or Lubrol PX (data not shown) suggests that these complexes represent the native state of the Rh polypeptides within the membrane, rather than an artificially induced aggregate (Moller *et al.*, 1988). These measurements

TABLE II

Physical properties of ^{3}H-palmitoylated Rh polypeptide membrane complex in 1% Triton X-100

Property	Value
Stokes radius, R_s[a]	52.4 Å
Sedimentation coefficient, $S_{20,w}$[b]	8.6 S
Partial specific volume, $\bar{v}$[c]	0.817 ml/g
Triton X-100 bound[d]	0.667 mg/mg protein
Molecular mass of detergent-protein complex[e]	282,000 daltons
Molecular mass of protein[c,e]	170,000 daltons
Molecular mass, SDS-PAGE[f]	32,000 daltons
Frictional ratio, f/f_0[e]	1.24

[a] Estimated by gel filtration (see Fig. 6*B*).

[b] Estimated by sedimentation on 5–20% sucrose gradients (see Fig. 6*A*).

[c] Calculated from sucrose gradients in H_2O and D_2O by method of Sadler (1979).

[d] Calculated assuming $\bar{v}_{protein} = 0.735$ (Smith, 1970) and $\bar{v}_{Triton\ X\text{-}100} = 0.94$ (Steele *et al.*, 1978).

[e] Calculated from the equations

$$M_r = \frac{6\pi N R_s S_{20,w}}{1 - \bar{v}\rho_{20,w}}$$

and

$$f/f_0 = R_s\left(\frac{4\pi N}{3M_r(\bar{v} + \delta\rho)}\right)^{1/3}$$

Where $N = 6.02 \times 10^{23}$ and δ was assumed to be 0.2 g solvent/g protein (Tanford, 1961) and $\rho_{20,w} = 0.9982$ g/ml.

[f] SDS-PAGE using buffers of Laemmli (1970).

permitted calculation of the apparent molecular weight of the detergent-Rh protein complex, $M_r = 282{,}000$ (Table II).

The apparent molecular weight of the Rh protein complex, $M_r = 170{,}000$, was estimated by correcting for detergent bound. It is likely that this value reflects imprecisions inherent in determining the physical size of a hydrophobic membrane protein; nevertheless, each of the analyses suggest that the Rh polypeptides exist in the membrane as a macromolecular complex.

Acknowledgments—We thank Daniel Raben for helpful suggestions and gas chromatography analyses and Barbara Smith for helpful discussions. We also thank Gerald Hart, Sally Craig, and Thomas Pollard for critical reading of the manuscript.

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