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Polymorphism in the M_r 32,000 Rh protein purified from Rh(D)-positive and -negative erythrocytes

(blood group antigen/erythrocyte membrane protein)

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ABSTRACT **A M_r 32,000 integral membrane protein has previously been identified on erythrocytes bearing the Rh(D) antigen and is thought to contain the antigenic variations responsible for the different Rh phenotypes. To study it on a biochemical level, a simple large-scale method was developed to purify the M_r 32,000 Rh protein from multiple units of Rh(D)-positive and -negative blood. Erythrocyte membrane vesicles were solubilized in $NaDodSO_4$, and a tracer of immunoprecipitated ^{125}I surface-labeled Rh protein was added. The Rh protein was purified to homogeneity by hydroxylapatite chromatography followed by preparative $NaDodSO_4$/PAGE. Approximately 25 nmol of pure Rh protein was recovered from each unit of Rh(D)-positive and -negative blood. Rh protein purified from both Rh phenotypes appeared similar by one-dimensional $NaDodSO_4$/PAGE, and the N-terminal amino acid sequences for the first 20 residues were identical. Rh proteins purified from Rh(D)-positive and -negative blood were compared by two-dimensional iodopeptide mapping after ^{125}I-labeling and α-chymotrypsin digestion. The peptide maps were very similar; however, at least two additional iodopeptides were consistently noted in the Rh proteins purified from Rh(D)-positive erythrocytes. These data indicate that a similar core Rh protein (or group of related proteins) exists in both Rh(D)-positive and -negative erythrocytes, and the Rh proteins from erythrocytes with different Rh phenotypes contain distinct structural polymorphisms.**

The human erythrocyte Rh blood group antigens and antibodies are of major clinical significance (for reviews, see refs. 1–3). In addition to being a large concern in transfusion medicine, Rh-specific antibodies have been implicated in most cases of hemolytic disease of the newborn and in many cases of autoimmune hemolytic anemia. The Rh blood group system is complex, and molecular understanding of this system has developed slowly. It remains uncertain whether the major Rh antigen (D) and the minor Rh antigens (C/c and E/e) are the products of a single gene (4) or multiple closely linked genes (5).

Major advances have resulted from the identification of a M_r 32,000 integral membrane protein on erythrocytes bearing the Rh(D) antigen. This protein (referred to hereafter as the "Rh protein") can be labeled on the extracellular surface of the erythrocyte membrane with ^{125}I and can be immunoprecipitated with Rh(D) immunoglobulin (6, 7). The Rh protein was found to contain no detectable carbohydrate (8) and is linked to the erythrocyte membrane skeleton (9, 10). The Rh protein can be disassociated from the membrane skeleton under more stringent conditions (11), and no cytoplasmic domain has been detected (12). Digestion of intact erythrocytes with high concentrations of proteases or glycosidases will destroy the surface domains of other erythrocyte membrane proteins, yet the Rh(D) protein survives (8, 12).

Reports describing the purification of small amounts of the Rh protein have recently appeared (11, 12). Several questions remain unanswered with regard to the relationship between the purified Rh protein and the Rh antigen on the intact membrane. Because of its limited solubility, it has been impossible to reinsert the purified Rh protein into lipid vesicles with immunologic restoration of the Rh antigen. While Rh immunoglobulin reacts avidly with Rh antigen on intact membranes, it fails to react specifically with Rh protein or other membrane components on immunoblots (12). If the Rh protein is the major constituent of the Rh antigen, there should be inherent biochemical differences in the Rh protein purified from erythrocytes of different Rh phenotypes. Determination of amino acid sequence of the Rh protein may ultimately permit molecular genetic analysis of this complex blood group system. Purification of larger amounts of the Rh protein will therefore be essential for molecular understanding of Rh biology.

MATERIALS AND METHODS

Purification of Rh Protein. Methods were derived from those described by Bennett (13) for large-scale preparation of membranes. Human blood was provided by the Johns Hopkins Hospital Blood Bank and the American Red Cross. Each preparation was begun with 1 full unit of human erythrocytes, and the quantity of membranes at each stage of isolation corresponds to this original volume of packed erythrocytes. The packed erythrocytes were filtered through a Sepacell R-500 leukocyte removal filter (Fenwal) and washed free of plasma and platelets in phosphate-buffered saline. Whole erythrocyte membranes were prepared by repeatedly lysing and washing in 7.5 mM sodium phosphate, pH 7.4/1 mM sodium EDTA/0.2 mM phenylmethylsulfonyl fluoride at 0°C with a Pellicon cassette system blood lysis apparatus (Millipore). Spectrin and actin were extracted from whole membranes by incubation in 10 vol of 0.2 mM sodium EDTA, pH 8.0/0.2 mM phenylmethylsulfonyl fluoride for 30 min at 37°C, and the membrane vesicles were pelleted by centrifugation at 30,000 × *g* in a Beckman J-14 rotor for >6 hr at 4°C. The membrane pellet was vesiculated by drawing through a 19-gauge syringe needle and further stripped of all peripheral membrane proteins by incubation in 10 vol of 1 M KI/7.5 mM sodium phosphate, pH 7.4/1 mM sodium EDTA/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride for 30 min at 37°C. The KI-stripped membrane vesicles were pelleted at 30,000 × *g* for 5 hr at 4°C, washed again with the same buffer once, and washed finally with 7.5 mM sodium phosphate, pH 7.4/1 mM dithiothreitol/1 mM NaN_3. The vesicle pellet was solubilized in 100 ml of 1.2% (wt/vol) $NaDodSO_4$/7.5 mM sodium phosphate, pH 7.4/1 mM

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dithiothreitol/1 mM NaN_3 by incubation for 1 hr at room temperature followed by filtration through 0.2-μm membranes (Millipore).

The tracer ^{125}I-labeled Rh protein was prepared by surface labeling whole Rh(D)-positive erythrocytes with carrier-free $Na^{125}I$ (Amersham) in vials coated with Iodo-Gen (Pierce) followed by immunoprecipitation with Rh(D) immunoglobulin (Connaught Laboratories, Willowdale, Ontario) and preparative $NaDodSO_4$/PAGE as described (12). Approximately 25,000 cpm of the ^{125}I-labeled Rh protein <0.1 μg of protein) was added to the solubilized vesicles.

The material was absorbed onto a column (2.5 × 62 cm) packed with high resolution hydroxylapatite (Calbiochem) that had previously been equilibrated with 0.2% (wt/vol) $NaDodSO_4$/7.5 mM sodium phosphate, pH 7.4/1 mM dithiothreitol/1 mM NaN_3 at a flow rate of 50 ml/hr while 8-ml fractions were collected (Fig. 1). The column was eluted with 0.3 M (peak I) and 0.5 M sodium phosphate (peak II) prior to elution with a 500-ml gradient of 0.5 to 0.8 M sodium phosphate in the same buffer while optical absorbance at 280 nm and ^{125}I radioactivity were monitored.

Fractions containing the peak and trailing side of the Rh protein were combined, dialyzed against the running buffer, and concentrated by shaking the dialysis bag in powdered polyethylene glycol (Carbowax, PEG 8000; Fisher). The contents of the bags were electrophoresed through 0.3 × 14 × 14 cm preparative 12% $NaDodSO_4$/polyacrylamide gels (Bio-Rad reagents) with the Laemmli buffer system (14). The gels were cut into narrow strips, assayed for ^{125}I, and the peak fractions were eluted with running buffer by gently shaking for 2 days at room temperature. The M_r 32,000 Rh protein was identified at each stage of isolation by silver staining analytical $NaDodSO_4$/polyacrylamide gels.

Amino Acid Sequencing. Approximately 30 μg of purified Rh proteins was precipitated with chilled ethyl alcohol. Determination of N-terminal amino acid sequences was performed in the Johns Hopkins Protein/Peptide Facility in the Department of Biological Chemistry with the Applied Biosystems gas-phase protein sequencer (model 470A) coupled to an on-line PTH analyzer (model 120A).

Two-Dimensional Iodopeptide Maps. Methods were derived from those of Elder *et al.* (15). Approximately 10 μg of purified Rh protein was precipitated in acetone (−20°C) and resolubilized by incubation for 1 hr at 37°C in 30 μl of 6% (wt/vol) $NaDodSO_4$/50 mM sodium phosphate, pH 7.4. The material was labeled with 1 mCi of carrier-free $Na^{125}I$ (1 Ci = 37 GBq) after oxidation with chloramine T (Kodak) as described (16). Approximately 50% of the radioactivity was incorporated when assessed by precipitation with trichloroacetic acid. The contents of each tube were run out on 12% $NaDodSO_4$/polyacrylamide gels and the radioactive spots corresponding to the ^{125}I-labeled Rh protein were visualized by autoradiography. Slices containing the ^{125}I-labeled Rh protein were excised, transferred to siliconized tubes, and washed extensively first with 25% isopropyl alcohol and then with 10% methyl alcohol. The slices were lyophilized and incubated at 37°C in 2.0 ml of 50 mM NH_4HCO_3 containing 100 μg of α-chymotrypsin (Cooper, Malvern, PA) for 15 hr. An additional 100 μg of α-chymotrypsin was added and the incubation was allowed to proceed for 9 hr more before the contents were lyophilized, redissolved in electrophoresis buffer (acetic acid/formic acid/water; 15:5:80), spotted on 20-cm cellulose thin-layer chromatogram sheets (Kodak), and electrophoresed at 1000 V for 45 min. The second dimension of separation was by thin-layer chromatography in butyl alcohol/pyridine/acetic acid/water (80:53:12:55). The dried plates were exposed to autoradiograph film.

RESULTS

Purification of the Rh Protein. Erythrocyte membranes were stripped of spectrin, actin, and other proteins, and the resulting membrane vesicles were further stripped of all peripheral membrane proteins by incubation in 1 M KI (13). These vesicles retain only proteins that penetrate the lipid bilayer (integral membrane proteins) including the glycophorins, the anion transporter, and the Rh protein (12). Because of the limited solubility of the Rh protein, the vesicles were solubilized in $NaDodSO_4$. Since the surface antigenicity is lost after solubilization of membranes, the Rh protein was followed during purification by adding a radiolabeled tracer of Rh protein immunoprecipitated from ^{125}I surface-labeled Rh(D)-positive erythrocytes with Rh(D) immunoglobulin (12).

The solubilized material was adsorbed onto a hydroxylapatite column and eluted with a sodium phosphate gradient (Figs. 1 and 2). The glycophorins (peak I) and the bulk of the anion transporter (peak II) eluted early. The elution behavior of the Rh protein was unique and constituted the majority of the protein eluted at the high end of the sodium phosphate gradient (fractions IIIB–D), although a small amount of anion transporter eluted similarly. The Rh protein was further

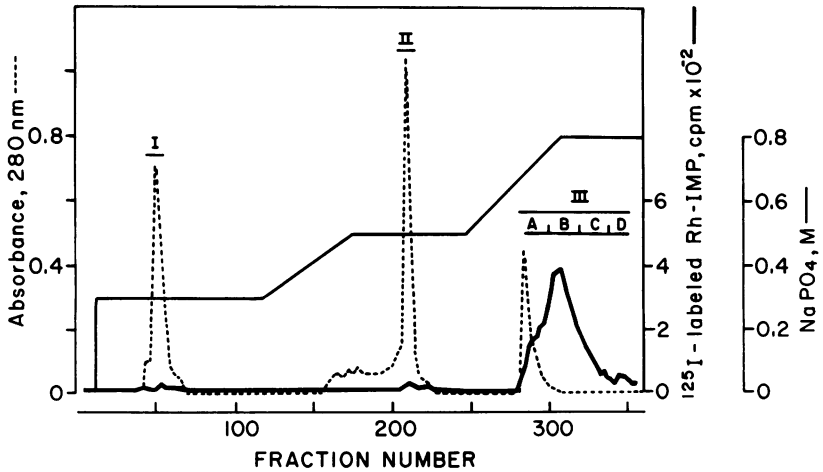

FIG. 1. Purification of the Rh protein by hydroxylapatite chromatography. Erythrocyte membrane vesicles from 1 unit of blood were solubilized in $NaDodSO_4$ and combined with a tracer of ^{125}I surface-labeled Rh protein. The material was loaded onto a hydroxylapatite column and eluted with sodium phosphate gradients while monitoring for optical absorbance at 280 nm and ^{125}I radioactivity. Contaminating membrane proteins were eluted at the lower sodium phosphate concentrations (peaks I and II), and a third protein peak (III) was eluted by the 0.5–0.8 M sodium phosphate gradient. The bulk of the ^{125}I-labeled Rh protein (fractions B and C) trailed after the major protein in peak III.

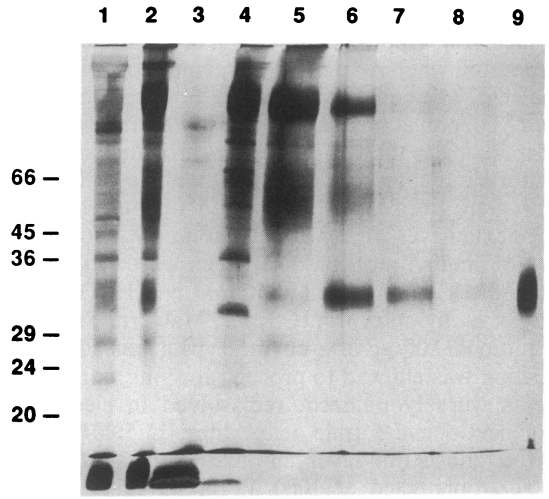

FIG. 2. Stages of the purification analyzed by 12% $NaDodSO_4$/PAGE after staining with silver reagent. Numerals on left indicate relative $M_r \times 10^{-3}$. Lanes: 1, whole erythrocyte membranes; 2, KI-extracted membrane vesicles. Remaining lanes refer to fractions from Fig. 1: lane 3, peak I; lane 4, peak II; lane 5, fraction IIIA; lane 6, fraction IIIB; lane 7, fraction IIIC; lane 8, fraction IIID; lane 9, pure Rh protein from fractions IIIB–D eluted from preparative $NaDodSO_4$/PAGE.

purified to homogeneity by elution from preparative $NaDodSO_4$/polyacrylamide gels (Fig. 2). Stages of a typical purification are shown in Table 1. Approximately 740 μg of pure Rh protein (20–25 nmol) was purified from a unit of blood, and this represented a yield of ≈19%, based on recovery of the tracer of ^{125}I-labeled Rh protein. Similar recoveries were noted in purifications of Rh protein from 8 units of blood from different Rh(D)-positive individuals. Evidence for purity of the isolated Rh protein includes the following: (*i*) A single band was seen on silver-stained $NaDodSO_4$/polyacrylamide gels. (*ii*) Aberrant migration of the single band was noted on gels of higher acrylamide concentration (data not shown; see also refs. 8 and 12). (*iii*) A single N-terminal amino acid sequence was identified (see below). It cannot be determined, however, whether the purified material is a mixture of nearly identical isoforms of the Rh protein.

Purification of Rh Protein from Rh(D)-Negative Erythrocytes. It was uncertain whether a protein comparable to the Rh protein would exist in Rh(D)-negative erythrocytes. Therefore, purification of the Rh protein was followed with a tracer of ^{125}I-labeled Rh protein immunoprecipitated from Rh(D)-positive erythrocytes. This method was used to purify the Rh protein from 5 units of blood from different Rh(D)-negative individuals. The yields from all Rh(D)-positive and -negative preparations were quantitatively similar (600–800 mg per unit of blood), and analysis by one-dimensional $NaDodSO_4$/PAGE demonstrated a heavy band at M_r 32,000 in each purification (Fig. 2).

Table 1. Purification of Rh protein from 1 unit of blood

Step	Protein, mg	Recovery ^{125}I, %	Purification, -fold	Relative purity, %
Whole membranes	700			
Membrane vesicles	220	100	3.2	
Hydroxylapatite	16	54	24.4	50
Preparative $NaDodSO_4$/PAGE	0.74	19	180	>95

Protein determinations were performed by the method of Lowry *et al.* (17), with appropriate buffer controls and bovine serum albumin used as standards. Relative purity was determined by densitometric scanning of $NaDodSO_4$/polyacrylamide gels stained with silver reagent.

N-Terminal Amino Acid Sequence of Purified Rh Proteins. The Rh proteins purified from pooled Rh(D)-negative erythrocytes obtained from four different individuals (probable genotypes *cde/cde*) were subjected to amino acid sequencing. Rh proteins purified from pooled Rh(D)-positive erythrocytes obtained from two individuals (probable genotypes *CDe/cDE*) were processed similarly. The N-terminal amino acid sequences were obtained up to the 20th residues, and the sequences of both preparations were identical: Ser-Ser-Lys-Tyr-Pro-Arg-Ser-Val-Arg-Arg-Xaa-Leu-Pro-Leu-Trp-Ala-Leu-Thr-Leu-Glu. When compared to the computer-based sequence library, it was found that this represents a unique amino acid sequence with no closely related amino acid sequences yet identified.

The initial amino acid recoveries obtained from sequencing the purified Rh(D)-negative protein were >90% of the anticipated values, but recoveries from sequencing the purified Rh(D)-positive protein were only 40%. It is uncertain whether the lower recovery is the result of a partial N-terminal block due to damage inflicted during isolation or a naturally occurring partial N-terminal block. Nevertheless, for each preparation, serine was unambiguously identified in the first two cycles with other amino acids accounting for <5% of the initial signal. Recoveries gradually diminished with each subsequent cycle to approximately half of the original recovery by the 20th cycle. The fact that unblocked N-terminal serines were identified suggests that the Rh proteins were derived from a larger protein by proteolytic cleavage after insertion into the lipid bilayer.

Comparison of Two-Dimensional Iodopeptide Maps. Rh proteins purified from the Rh(D)-positive and -negative erythrocytes were precipitated with cold acetone, labeled to high specific radioactivity with ^{125}I, and digested with α-chymotrypsin (15). The iodopeptides were compared in two dimensions by electrophoresis followed by thin-layer chromatography (Fig. 3). The two-dimensional iodopeptide maps of Rh proteins purified from Rh(D)-positive and -negative erythrocytes were very similar, with approximately one dozen iodopeptides appearing to be identical. At least two additional iodopeptides were identified in maps of Rh proteins purified from Rh(D)-positive erythrocytes. Two distinct iodopeptides were noted (nearest the center of the peptide map), and two less distinct peptides were also detected (at the far right of the peptide map). The two central iodopeptides appear highly specific for Rh proteins from Rh(D)-positive erythrocytes and have been seen in every iodopeptide map of all preparations from Rh(D)-positive erythrocytes obtained from eight individuals but have never been noted in preparations from Rh(D)-negative erythrocytes obtained from five individuals.

DISCUSSION

It is likely that information derived from analysis of the Rh protein may lead to a clearer molecular understanding of the Rh antigen. The method described in this study will permit the isolation of large amounts of Rh protein in pure form. This method is much simpler than that recently reported for purification of Rh protein beginning with membrane skeletons, and the yields are ≈8-fold larger (12). The Rh protein has recently been purified on a smaller scale by monoclonal antibody immunoprecipitations (11), and sequence analyses of those preparations agree with that reported here (18). Such large-scale purifications will be needed to provide sufficient pure Rh protein to permit biochemical analysis of possible posttranslational modifications and for generation of internal peptide fragments to obtain internal amino acid sequences. Additional sequences may be very important for develop-

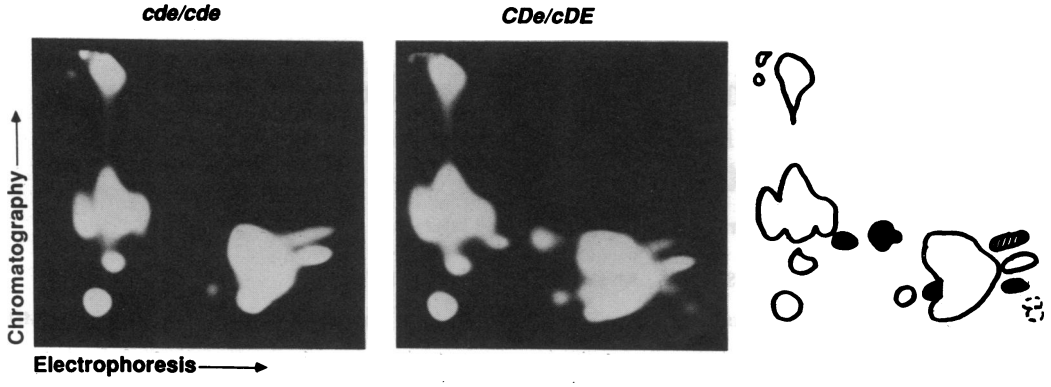

FIG. 3. Rh proteins purified from Rh(D)-negative (*cde/cde*; *Left*) and -positive (*CDe/cDE*; *Center*) erythrocytes ere compared by two-dimensional iodopeptide mapping. The composite drawing (*Right*) identifies peptides shared by Rh proteins from both sources (open figures). Four peptides are identified that are unique to Rh protein purified from Rh(D)-positive erythrocytes (solid figures), and a single peptide was found partially reduced in Rh protein from Rh(D)-positive erythrocytes (hatched figure).

ment of the oligonucleotide probes needed for isolating the Rh cDNA, since most of the sequence reported here contains amino acids for which the genetic code is most degenerate.

Isolation of the Rh cDNA will be very desirable, since ultimate determination of the complete amino acid sequence of the Rh antigens will very likely require molecular genetic analyses. Despite four and one-half decades of investigation, it remains uncertain whether the Rh C/c, D, and E/e antigens are the products of a single gene (4) or multiple closely linked genes (5). Existence of d, the antithetical antigen to Rh(D), is speculative and has never been confirmed. It remains to be determined whether the Rh protein purified in these studies is composed of a single protein with multiple surface epitopes or a mixture of distinct C, c, D, E, and e proteins. There appears to be only a single protein band of M_r ≈32,000, and the amino acid sequences were unambiguous. Therefore, if multiple distinct protein species are present, they must be very similar: identical N termini, nearly identical molecular weights, and identical elution behavior from hydroxylapatite. Immunoprecipitations from ^{125}I surface-labeled erythrocytes with antibodies specific for c, D, or E, resulted in isolation of similar M_r 30,000 proteins with differences in specific radioactivities (6). Very recent work (19) indicates that immunoprecipitated c and E proteins are slightly larger (M_r, 33,100) than the D protein (M_r, 31,900), and one-dimensional iodopeptide maps of immunoprecipitated Rh c, D, and E proteins indicate that they are similar, but not identical (18). These observations suggest that the C/c, D, and E/e antigens may be variations of the same gene product that results either from gene duplication or alternate splicing of mRNA leading to regions of nonidentity. There are at least two possible explanations for the apparent absence of certain iodopeptides from Rh(D)-negative preparations without the appearance of new iodopeptides. (*i*) There may be point mutations where tyrosines are replaced by other amino acids. (*ii*) There may be alternate splicing of mRNA with exons containing tyrosine being replaced by exons lacking tyrosine.

Explanation of a possible physiologic role for the Rh protein has yet to be established. A rare group of individuals, described as "Rh null," lack all Rh antigens on their erythrocytes; C, c, D, E, e, and Rh29 are not detected (20). The erythrocytes from these patients are abnormally shaped and fragile. It is thought that the Rh antigen may therefore have a structural role. Identification of the structural association of the Rh protein with the membrane skeleton suggests that it may be involved in a secondary form of linkage of the skeleton to the lipid bilayer (9, 10). The phospholipid asymmetry of the bilayer is lost from the membranes of the Rh null erythrocytes (21), and a variety of ion leaks have also been detected (22). Direct involvement of the Rh protein in maintenance of phospholipid asymmetry and the appropriate lipid milieu for ion transporters and channels have been considered as other potential roles of the Rh protein. The identification of nonerythroid analogs to all other erythrocyte structural proteins suggests that analogs to the Rh protein (which may lack the surface antigenic component) may also exist and could be identified with Rh cDNA probes.

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