MOLECULAR IDENTIFICATION OF THE HUMAN Rh_o(D) ANTIGEN

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1. Introduction

The molecular nature of the Rh_o(D) antigen of the human erythrocyte blood group has remained most controversial. Although several studies agree that the antigen is an integral membrane protein, M_r estimates vary from 7000–180 000 [1–4]. We have now radioactively surface-labeled Rh_o(D) positive and negative erythrocytes using the ^{125}I/lactoperoxidase method. The labeled membrane proteins were solubilized in Triton X-100-containing buffer and immunoprecipitated with anti-Rh_o(D) antisera. The precipitates were analysed by sodium dodecyl sulfate (SDS)–polyacrylamide slab gel electrophoresis. The results show that only from Rh_o(D) positive membranes a single minor polypeptide with app. M_r 28 000–33 000 could be precipitated specifically. Its behavior during electrophoresis indicated a hydrophobic nature. It may lack carbohydrate because it was not labeled using carbohydrate-specific surface labeling techniques.

2. Materials and methods

Human red cells were Rh_o(D) typed by standard techniques. The red cells and the anti-Rh_o(D) specific antisera were obtained from the Finnish Red Cross Blood Transfusion Centre, Helsinki. The red cells were radioactively labeled by lactoperoxidase-catalyzed iodination in the presence of D-glucose and glucose oxidase [5]. The membranes were isolated [6] and solubilized in 0.15 M NaCl–0.01 M sodium phosphate (pH 7.4)–1% Triton X-100–1% ethanol–2 mM phenylmethylsulfonyl fluoride at 0°C. All subsequent operations until denaturation with SDS were done at 0–4°C. After centrifugation at 5000 rev./min for 10 min, the supernatants were recovered and subjected to immunoprecipitations. These were performed using protein A-containing *Staphylococcus aureus* cells as detailed in [7]. SDS–polyacrylamide slab gel electrophoresis was done as in [8]. After electrophoresis the gels were fixed, treated for fluorography [9] and exposed to Kodak RP X-Omat film. ^{14}C-Labeled standard proteins were obtained from the Radiochemical Centre, Amersham. The app. M_r of the Rh_o(D) antigen was determined as in [10]. Red cells were labeled by the periodate/NaB^3H_4 and neuraminidase–galactose oxidase/NaB^3H_4 methods as in [6,11].

3. Results

Fig.1 shows a polyacrylamide slab gel of erythrocyte surface proteins and immunoprecipitates obtained with anti-Rh_o(D) antiserum. A surface-labeled protein (marked with the heavy arrow) was specifically precipitated from the Rh_o(D) positive membranes (fig.1D,F,H) with antiserum. Non-immune sera did not precipitate the polypeptide (fig.1E,G,I), neither did antiserum from Rh_o(D) negative membranes (fig.1J,L). The app. M_r of the Rh_o(D) antigen on 12% acrylamide gel was 33 000. When immunoprecipitates were analyzed using 8% acrylamide gels, the app. M_r was 28 000 (fig.2D). An identical polypeptide was obtained from En(a–) Rh_o(D) positive membranes (fig.2F), which lack glycophorin A and contain an overglycosylated band 3 [12–14].

The Rh_o(D) antigen is evidently a minor component of the membrane. When ^{125}I-labeled membranes were directly analyzed by polyacrylamide slab-gel electrophoresis followed by fluorography no labeled polypeptide was seen in the position of the Rh_o(D) antigen after a relatively short exposure to X-ray film (fig.3B).

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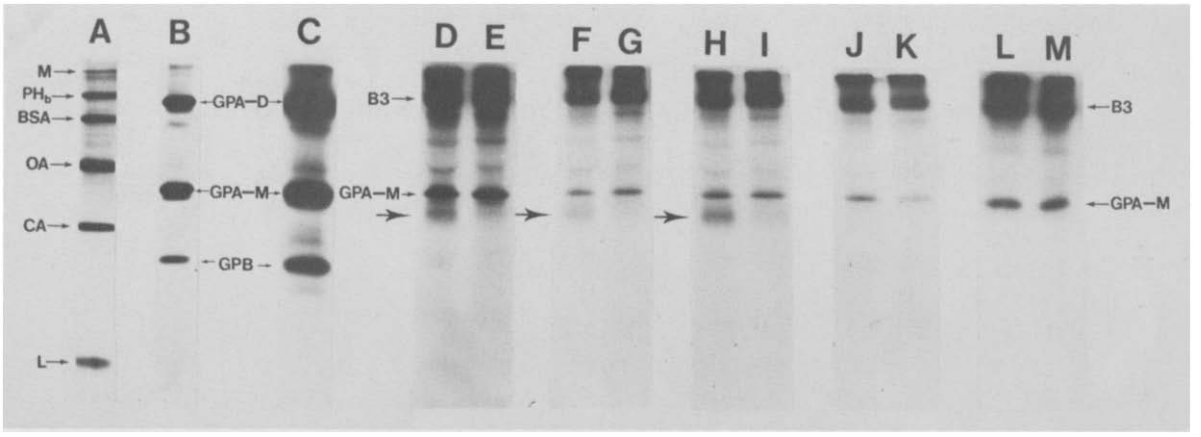

Fig.1. Fluorography pattern of a 12% polyacrylamide slab gel of immunoprecipitated $Rh_o(D)$ antigens from ^{125}I-labeled red cell membranes: (A) ^{14}C-labeled standard proteins [M, myosin; PH_b, phosphorylase *b*; BSA, bovine serum albumin; OA, ovalbumin; CA, carbonic anhydrase; L, lysozyme]; (B) pattern of periodate/NaB^3H_4-labeled red cell membranes [GPA-D, glycophorin A dimer; GPA-M, glycophorin A monomer; GPB, glycophorin B]; (C) pattern of ^{125}I-labeled red cell membranes; (D,F,H) patterns obtained by immunoprecipitation with anti-$Rh_o(D)$ antiserum from $Rh_o(D)$ positive red cell membranes; B3, band 3 (nomenclature from [23]). Heavy arrow points to the position of the $Rh_o(D)$ antigen; (E,G,I) patterns obtained with non-immune sera from identical samples as in (D,F,H); (J,L) patterns obtained with antiserum from $Rh_o(D)$ negative red cell membranes; (K,M) patterns obtained with non-immune serum from identical samples as in (J,L).

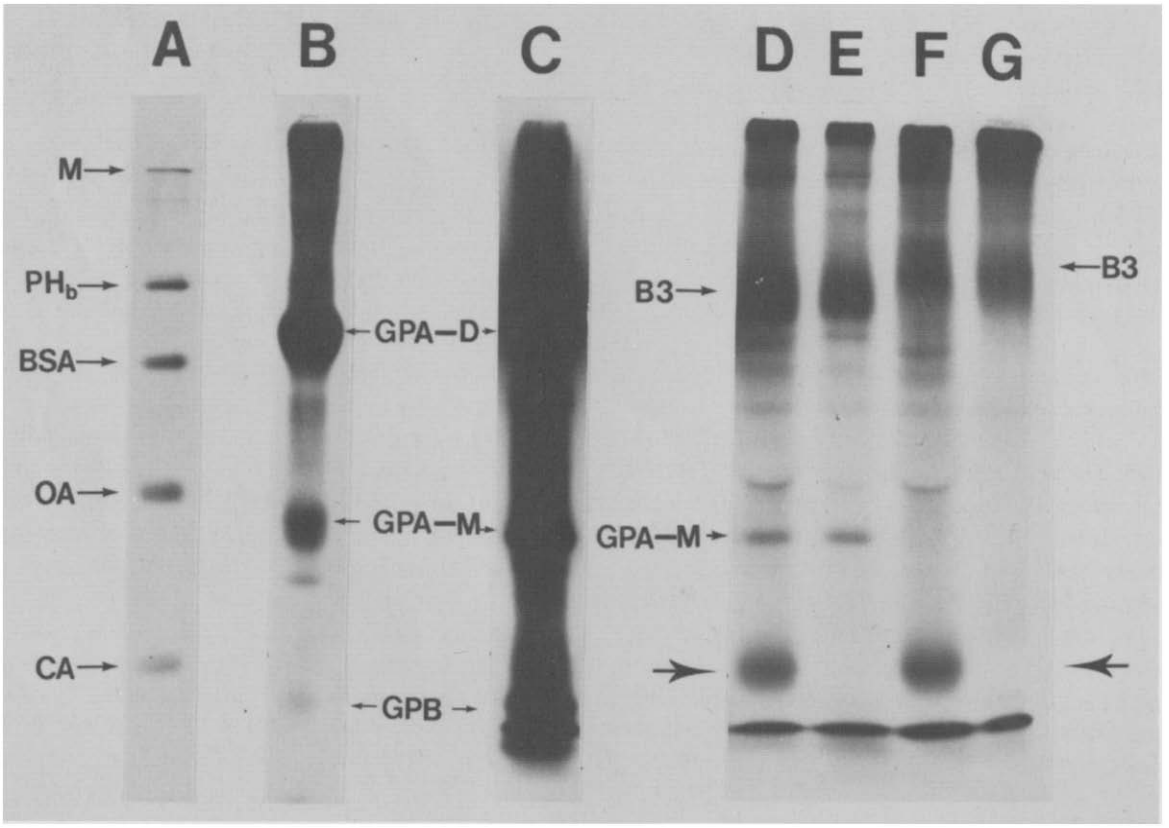

Fig.2.

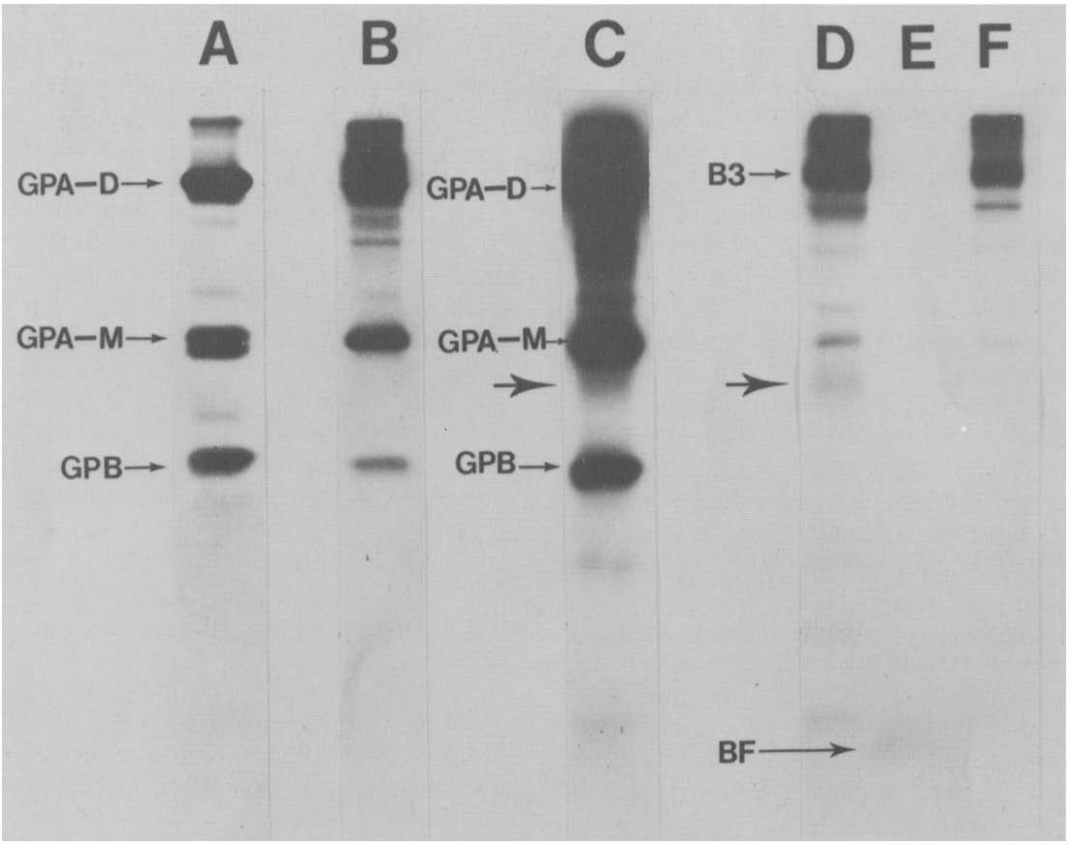

Fig.3. Fluorography pattern of a 12% slab gel of surface-labeled red cells and $Rh_0(D)$ antigen treated with pronase: (A) pattern of periodate/NaB^3H_4-labeled red cell membranes; (B) pattern of ^{125}I-labeled $Rh_0(D)$ positive red cell membranes; (C) as in (B) but exposed to X-ray film for a longer time; (D) patterns obtained with anti-$Rh_0(D)$ antiserum from $Rh_0(D)$ positive ^{125}I-labeled membranes; (E) an identical immunoprecipitate as in (D) which was treated subsequently with 1% pronase in 1% SDS for 1 h at 37°C; (F) pattern obtained with non-immune serum from an identical sample as in (D). Symbols as in legend to fig.1; (➡) $Rh_0(D)$ antigen.

A longer exposure visualized a labeled component in that position (fig.3C, arrow). The protein nature of the antigen was ascertained by protease digestion. Fig.3D shows the immunoprecipitated antigen, and it was completely degraded by pronase (fig.3E). Only some low M_r peptides remained in the region of the buffer front (BF).

4. Discussion

Here I have shown that a surface-exposed polypeptide with an app. M_r 28 000–33 000 was specifically immunoprecipitated from $Rh_0(D)$ positive red cell membranes using anti-$Rh_0(D)$ antiserum. The polypeptide has now been immunoprecipitated from

Fig.2. Fluorography pattern of an 8% polyacrylamide slab gel of $Rh_0(D)$ antigens immunoprecipitated from normal ^{125}I-labeled $Rh_0(D)$ positive red cell membranes and $Rh_0(D)$ positive En(a–) red cell membranes: (A) standard proteins, see legend to fig.1; (B) pattern of periodate/NaB^3H_4-labeled red cell membranes; (C) pattern of ^{125}I-labeled $Rh_0(D)$ positive red cell membranes; (D) pattern obtained with anti-$Rh_0(D)$ antiserum from $Rh_0(D)$ positive red cell membranes; (E) pattern obtained with non-immune serum from an identical sample as in (D); (F) pattern obtained with antiserum from $Rh_0(D)$ positive En(a–) red cell membranes; (G) pattern obtained with non-immune serum from an identical sample as in (F). Symbols as in legend to fig.1. (➡) $Rh_0(D)$ antigen. The weakly labeled band just above GPA-M in D and F is actin, which non-specifically may bind to immunoprecipitates.

>20 $Rh_o(D)$ positive cell samples but has never been obtained from $Rh_o(D)$ negative cells. Several polypeptides were non-specifically adsorbed to the Staphylococci, among them glycophorin A and a protein migrating in the band 3 position. These are the most strongly labeled surface polypeptides of red cells and evidently represent 'background' radioactivity.

The app. M_r of the $Rh_o(D)$ polypeptide was 28 000 using an 8% acrylamide slab gel whereas the app. M_r was 33 000 using a 12% gel. The major red cell sialoglycoprotein, glycophorin A, which binds relatively low amounts of SDS [15,16] and is relatively hydrophilic due to its 60% content of carbohydrate gave a higher app. M_r on the 8% acrylamide gel than on the 12% gel (cf. fig.1D,2D). Thus the $Rh_o(D)$ antigen migrated near the glycophorin A monomer on 12% gels but much faster on 8% gels. This indicated that the $Rh_o(D)$ antigen binds relatively more SDS and is more hydrophobic [17].

The antigen is weakly labeled in the intact cell which indicates that it is a minor component. Previous quantitation has given values of 20 000–32 000 antigenic sites/cell [18]. In contrast some major components like glycophorin A and band 3 are found in 10^6 copies/cell [19,20]. But the $Rh_o(D)$ antigen may well be deeply embedded in the membrane and thus rather inaccessible to external reagents. In fact, protease treatment does not destroy the antigen in intact cells [21,22], whereas the isolated polypeptide was readily degraded.

We have performed immunoprecipitations with anti-$Rh_o(D)$ antisera from solubilized red cell membranes labeled by the galactose oxidase/NaB^3H_4 or periodate/NaB^3H_4 surface-labeling techniques, but no labeled band was obtained. It may be that the protein, although surface-located, does not contain carbohydrate.

Previous work on the molecular characterization of the $Rh_o(D)$ antigen has given variable results. Early work was hampered by the lack of suitable techniques for the characterization of integral membrane proteins. In [3] an antigenically active fraction was isolated by elution from anti-$Rh_o(D)$ IgG–Sepharose columns. After staining with Coomassie brilliant blue they observed a diffuse band migrating near the buffer front on SDS gel electrophoresis. The est. M_r was 7000. Unfortunately, the affinity chromatography was performed at room temperature, which could have resulted in proteolysis. Another possibility is that the eluate indeed contained the intact antigen, but it was not stained due to its hydrophobic nature.

In [4] it was claimed that the anion transport protein band 3 carries the $Rh_o(D)$ antigen activity. Their conclusion was based on the adsorption of band 3 molecules to anti-$Rh_o(D)$ IgG columns. The results are difficult to interpret because the band 3 polypeptides were extensively fragmented. In addition it is known that band 3 is easily aggregated [23,24] and a diminution in the gel profile of the eluted controls is not conclusive. Furthermore, the number of band 3 molecules found per cell does not agree with the estimation of the number of $Rh_o(D)$ antigenic sites.

This identification of the $Rh_o(D)$ polypeptide should facilitate its large scale purification. Antiserum production by immunization with the purified antigen would be highly desirable and may then become possible.

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