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Characterization of human red cell Rh (Rhesus-)specific polypeptides by limited proteolysis

Martina Krahmer and Rainer Prohaska

Institute of Biochemistry, University of Vienna, Währingerstrasse 17, A-1090 Vienna, Austria

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Human red cells of various Rh phenotypes were surface-labelled with ^{125}I and the Rh-specific labelled polypeptides were isolated by preparative SDS-PAGE. The polypeptides were subjected to limited proteolysis and the resulting fragments were analysed by SDS-PAGE and autoradiography. Chymotryptic peptide maps of proteins obtained from Rh(D)-positive and -negative types appeared completely identical, whereas tryptic peptide maps revealed a difference: a fragment of M_r 17500 was associated with the Rh(D) antigen, and one of M_r 19000 with the Rh(C/c,E/e) antigens. Treatment of Rh polypeptides with carboxypeptidase Y prior to tryptic digestion resulted in a shift of nearly all tryptic fragments, including a fragment of M_r 8000, indicating that the surface label was incorporated into the C-terminal part of the molecule.

Erythrocyte; Membrane protein; Rh antigen; Rhesus polypeptide; Peptide mapping

1. INTRODUCTION

The blood group Rhesus (Rh) antigens of human red cells [1,2] have recently been characterized as integral membrane proteins of apparent M_r ~30000 [3–8]. The Rh(D) polypeptide was found to be very hydrophobic and seemed to lack carbohydrate residues [5]. Moreover, the Rh(D) protein was shown to be linked to the red cell membrane skeleton [9–11]. Using special electrophoretic conditions the Rh(D) polypeptide was found to be distinct from Rh(c,E) polypeptide(s) [7], thereby supporting the two-locus theory for the genetic control of Rh antigen expression [12]. We present evidence from proteolysis studies that the Rh(D) and Rh(C/c,E/e) polypeptides are distinct, but highly homologous proteins.

Correspondence address: R. Prohaska, Institut für Biochemie, Universität Wien, Währingerstr. 17, A-1090 Wien, Austria

Abbreviations: PAGE, polyacrylamide gel electrophoresis; TPCK, 1-chloro-3-tosylamido-4-phenylbutan-2-one

2. MATERIALS AND METHODS

2.1. *Materials*

Rh-typed human red cells were kindly provided by the Institut für Blutgruppenserologie, University of Vienna. Red cell samples of rare Rh phenotypes were gifts from Drs C.C. Entwistle (Oxford), R.Y. Harding (Toronto), P.D. Issitt (Miami), O. Jensson (Reykjavik), and S. Müller (Düdingen). Antisera were from Behringwerke and Ortho, ^{125}I from Amersham, α-chymotrypsin and TPCK-trypsin from Serva, and carboxypeptidase Y from Sigma. Analytical grade reagents were from Serva and Merck.

2.2. *Labelling of red cells and identification of Rh-specific polypeptides*

Rh-typed human erythrocytes were ^{125}I-labelled (1 mCi/0.2 ml packed cells) by the Iodo-Gen

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method [13]. The membranes were isolated and immunoprecipitation of Rh antigens carried out as in [3,5].

2.3. *Purification of Rh-specific polypeptides*

Rh proteins were purified from ^{125}I-labelled red cell membranes (equivalent to 1 ml packed red cells) by preparative SDS-PAGE [14] (10% acrylamide). The gels were exposed to Kodak XAR film and the Rh-specific bands excised according to the autoradiogram. Electrophoretically eluted [15] Rh proteins were further purified by a second step of SDS-PAGE (17% acrylamide), electrophoretic elution and dialysis. The Rh proteins were then adsorbed to phenyl-Sepharose CL-4B (2 × 1 cm, equilibrated with 10 mM Tris-Cl, pH 7.5). After washing with 20 ml equilibrating buffer the column was eluted with 20 ml of 0.5% SDS in equilibrating buffer. The fractions containing radioactivity were pooled, dialysed against 1 mM EDTA and 0.05% NaN_3 and lyophilised.

2.4. *Limited proteolysis of Rh-specific polypeptides*

Lyophilised Rh proteins (~10000 cpm) were dissolved in 40 μl of 20 mM Tris-Cl, pH 7.5, and 10 μl protease solution (containing 1–10 μg protease) were added. The mixture was incubated for 2–5 h at 37°C and the reaction terminated by addition of 15 μl of 5-fold sample buffer [14] and heating for 3 min at 100°C. The peptides were separated by SDS-PAGE (17% acrylamide); the gels were dried and exposed to Kodak XAR film.

2.5. *Carboxypeptidase Y digestion of Rh-specific polypeptides*

Lyophilised Rh proteins (~10000 cpm) were dissolved in 40 μl of 20 mM *N*-ethylmorpholine acetate buffer, pH 7.0, and 10 μl carboxypeptidase Y solution (0.1 mg/ml) were added. The mixture was incubated for 12 h at 37°C, the reaction being terminated by heating for 3 min at 100°C followed by lyophilisation.

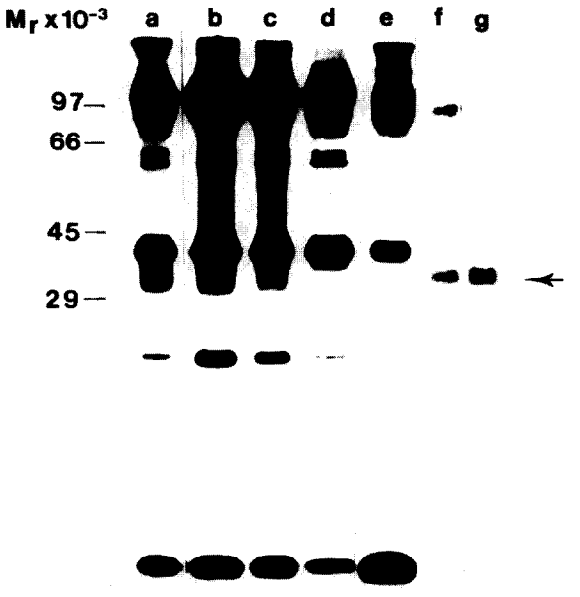

Fig.1. SDS-PAGE patterns of ^{125}I-labelled red cell membranes and of purified Rh-specific polypeptides. (a) Super-D (-D-/-D-) membranes, (b) cDE/cDE membranes, (c) cDe/cde membranes, (d) cde/cde membranes, (e) Rh_{null} (---/---) membranes, (f) immunoprecipitated Rh(D) protein, obtained from Super-D membranes with anti-Rh(D) antiserum, (g) purified Rh polypeptide(s) from cde/cde membranes. The arrow indicates the position of Rh-specific proteins. Acrylamide concentration, 12%.

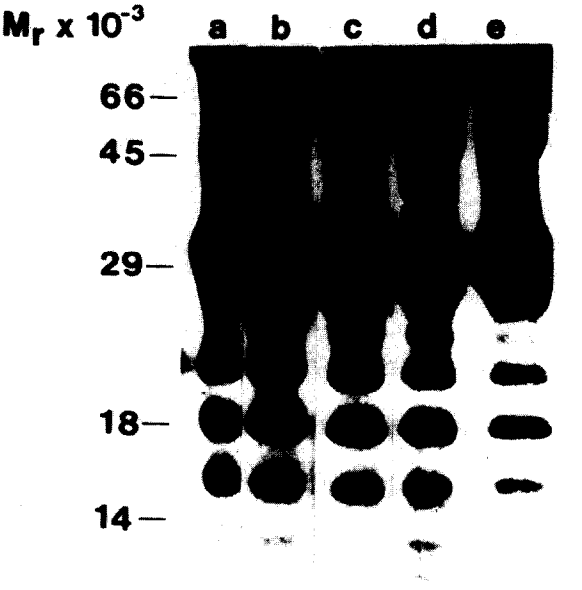

Fig.2. Peptide maps obtained from chymotryptic digests of purified Rh proteins from the phenotypes (a) -D-/-D-, (b) CDe/CDe, (c) cDE/cDE, (d) cDe/cde, (e) cde/cde. Acrylamide concentration, 17%.

3. RESULTS AND DISCUSSION

3.1. *Purification of Rh-specific polypeptides*

The Rh-specific proteins of M_r ~30000 were easily recognized when ^{125}I-labelled red cell membranes of different Rh phenotypes were analysed by SDS-PAGE, as described by others [3–6] (fig.1). Rh(D)-positive membranes showed a strongly labelled band of M_r ~30000, whereas this band was visible only after prolonged exposure when Rh(D)-negative (cde/cde) membranes were analysed. Rh_{null} membranes lacked the band of M_r ~ 30000.

We purified the Rh-specific proteins via two steps of preparative SDS-PAGE. 10% acrylamide gels gave optimal separation of Rh proteins from the glycophorins, 17% acrylamide gels being used for further purification from minor contaminants. Phenyl-Sepharose columns were used for the separation of Rh proteins from electrophoresis chemicals. The isolated Rh proteins showed the characteristic properties, as described [5], and co-migrated with immunoprecipitated Rh protein (fig.1).

3.2. *Peptide mapping of Rh-specific polypeptides*

Rh-specific proteins were digested with chymotrypsin or trypsin under conditions of limited proteolysis and were analysed by SDS-PAGE. The chymotryptic peptide maps of Rh proteins from Rh(D)-positive and -negative types appeared to be completely identical, indicating a high degree of structural homology between these proteins (fig.2).

In contrast, the tryptic peptide maps revealed a characteristic difference: a fragment of M_r 17500 was associated with the Rh(D) antigen and one of M_r 19000 with the Rh(C/c,E/e) antigens (fig.3). This result supports other evidence that the Rh(D)

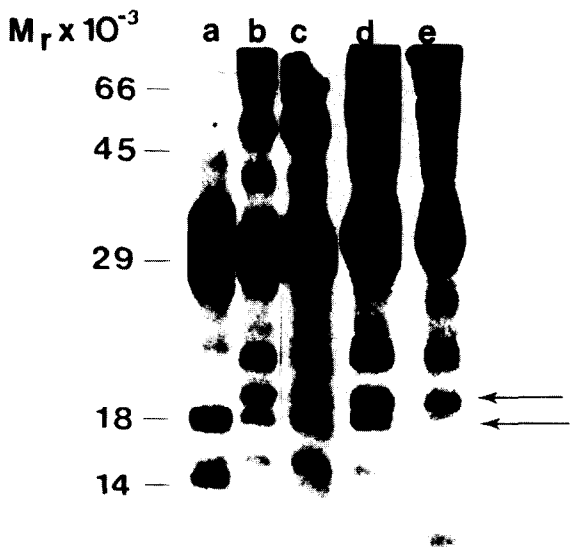

Fig.3. Peptide maps obtained from tryptic digests of purified Rh proteins from the phenotypes (a) -D-/-D-, (b) CDe/CDe, (c) cDE/cDE, (d) cDe/cde, (e) cde/cde. Arrows indicate the positions of the two characteristic fragments described in the text. Acrylamide concentration, 17%.

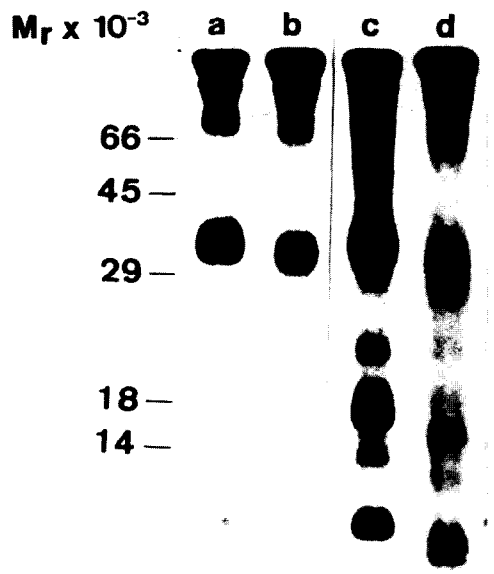

Fig.4. Peptide maps obtained from tryptic digests of purified cDe/cde protein(s), with or without prior treatment with carboxypeptidase Y. (a) Purified protein(s), (b) carboxypeptidase Y-digested protein(s), (c) tryptic peptide map of purified protein(s), (d) tryptic peptide map of carboxypeptidase Y-digested protein(s). Acrylamide concentration, 17%.

protein is not identical with the Rh(C/c,E/e) protein(s) [3,7,12]. Chemical modification studies involving arginine or lysine residues [16,17] in combination with tryptic peptide mapping showed that the Rh(D)-specific fragment is generated by cleavage at an arginine residue, whereas the fragment of M_r 19000 is generated by cleavage at a lysine residue (not shown).

3.3. *Peptide mapping of carboxypeptidase Y-digested Rh-specific polypeptides*

Incubation of Rh proteins with carboxypeptidase Y resulted in degradation of the polypeptide chains showing a decrease in M_r value of 1500 (fig.4). The tryptic peptide maps of untreated and carboxypeptidase Y-treated Rh proteins also exhibited a decrease in M_r value of 1500 for nearly all fragments, including one of M_r 8000. This result indicates that the surface label was incorporated into the C-terminal part of the molecules, suggesting further that the exposed Rh-specific epitopes are located in this region. However, we cannot exclude the possibility that other characteristic portions of the Rh proteins are also exposed at the cell surface, but are not ^{125}I-labelled. Alternative labelling methods should help to clarify this problem.

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