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Protein-sequence studies on Rh-related polypeptides suggest the presence of at least two groups of proteins which associate in the human red-cell membrane

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The Rh D blood-group antigen forms part of a complex, involving several other polypeptides, that is deficient in the red cells of individuals who lack all the antigens of the Rh blood-group system (Rh_{null} red cells). These include components recognized by anti-(Rh D) antibodies and the murine monoclonal antibodies R6A and BRIC 125. We have carried out protein-sequence studies on the components immunoprecipitated by these antibodies. Anti-(Rh D) antibodies immunoprecipitate an M_r-30000–32000 polypeptide (the D_{30} polypeptide) and an M_r-45000–100000 glycoprotein (D_{50} polypeptide). Antibody R6A immunoprecipitates two glycoproteins of M_r 31000–34000 ($R6A_{32}$ polypeptide) and M_r 35000–52000 ($R6A_{45}$ polypeptide). The D_{30} and $R6A_{32}$ polypeptides were found to have the same *N*-terminal amino acid sequences, showing that they are closely related proteins. The D_{50} polypeptide and the $R6A_{45}$ polypeptide also had indistinguishable *N*-terminal amino acid sequences that differed from that of the D_{30} and $R6A_{32}$ polypeptides. The putative *N*-terminal membrane-spanning segments of the two groups of proteins showed homology in their amino acid sequence, which may account for the association of each of the pairs of proteins during co-precipitation by the antibodies. Supplementary data related to the protein sequence have been deposited as Supplementary Publication SUP 50417 (6 pages) at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1988) **249**, 5.

INTRODUCTION

The Rh D blood-group antigen (often described as the 'rhesus D antigen') is of major clinical importance. The antigen has been shown to be associated with a red-cell membrane polypeptide of M_r 30000–32000 (Gahmberg, 1982; Moore *et al.*, 1982; denoted here as the 'D_{30} polypeptide'); however, the structure of the antigen remains unknown. Studies of membranes from the red cells of rare individuals who lack all of the antigens of the Rh blood-group system (Rh_{null} membranes) have suggested that the D polypeptide is part of a complex involving several other polypeptides (Moore & Green; 1987; Avent *et al.*, 1988). Immunoprecipitation experiments using anti-D antibodies have shown that a glycoprotein of M_r 45000–100000 (denoted here 'D_{50} polypeptide') co-precipitates with the D_{30} polypeptide (Moore & Green, 1987). Similar experiments using a murine monoclonal antibody (R6A) have shown that two further polypeptides, which also co-precipitate, appear to be deficient in Rh_{null} cells. Both polypeptides are glycosylated and have apparent M_r values of 31000–34000 (denoted here '$R6A_{32}$ polypeptide') and 35000–52000 (denoted here '$R6A_{45}$ polypeptide'; Ridgwell *et al.*, 1983; Avent *et al.*, 1988). Two glycoproteins of similar M_r are immunoprecipitated by both anti-c and anti-E antisera (Moore *et al.*, 1982; Moore & Green, 1987). Rh_{null} red cells are deficient in two further polypeptides defined by a murine monoclonal antibody, BRIC 125, and by anti-LW^{ab} antibodies (Moore, 1983; Mallinson *et al.*, 1986; Avent *et al.*, 1988). The BRIC 125 polypeptide is a glycoprotein of M_r 47000–52000, and the LW polypeptide(s) is a glycoprotein of M_r 40000–47000. There is further evidence that the polypeptide carrying the Rh blood-group antigens may form a complex with the minor red-cell membrane sialoglycoprotein δ (synonym glycophorin B; Dahr *et al.*, 1987).

We report here the results of amino-acid-sequence studies on the polypeptides precipitated by anti-D and R6A and show that the D_{30} polypeptide and the $R6A_{32}$ polypeptide have the same sequences at their *N*-termini. The D_{50} polypeptide and the $R6A_{45}$ polypeptide also have the same sequences at their *N*-termini, but these sequences are quite distinct from those of the D_{30} polypeptide and the $R6A_{32}$ polypeptide.

MATERIALS AND METHODS

Purification of the anti-Rh D and R6A binding polypeptides

Polypeptide material was purified essentially by a scaled-up form of the immunoprecipitation method of Moore *et al.* (1982), with the following modifications.

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Washed erythrocytes (100–200 ml) (Rh phenotype CDe) were incubated with either 1500 ml of culture supernatant containing approx. 10 μg of IgG_1/ml (human monoclonal anti-D (Kumpel *et al.*, 1988) or 5–10 ml of murine ascitic fluid (BRIC 69 or BRIC 207). BRIC 207 was produced and characterized as described for BRIC 69 (Avent *et al.*, 1988). After incubation with BRIC 69 or BRIC 207 the washed red cells were further incubated with rabbit anti-mouse IgG (Z109; Dakopatts, Copenhagen, Denmark). Immune complexes were solubilized in 5% (w/v) SDS/5 mM-EDTA/20 mM-Tris/HCl (pH 8.0)/10% (v/v) glycerol/Bromophenol Blue (0.1 mg/ml)/xylene xylol (0.1 mg/ml)/2 mM-phenylmethanesulphonyl fluoride, heated at 50 °C for 15 min and then separated on 10%-(w/v)-polyacrylamide gels using the buffer system of Laemmli (1970) with 1 mM-sodium thioglycollate added to the top chamber of the electrophoresis apparatus. Polypeptide material was localized either by autoradiography (by adding a small amount of immunoprecipitate from lactoperoxidase-radioiodinated red cells; Moore *et al.*, 1982), or by staining guide strips with Coomassie Brilliant Blue. Polypeptide material was then electroeluted from the gel slices essentially as described by Hunkapiller *et al.* (1983), dialysed against 0.1% (w/v) SDS, and then against water at 4 °C. Any remaining buffer salts were then removed by a single-solvent precipitation of the protein sample (Wessel & Flugge, 1984). The protein sequencing was done using a pulsed liquid-phase sequencer (model 477A; Applied Biosystems International). Supplementary data related to the protein sequence have been deposited as Supplementary Publication SU 50417 (6 pages) at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1988) **249**, 5.

RESULTS AND DISCUSSION

N-Terminal amino acid sequences of the D_{30} and D_{50} polypeptides

The D_{30} and D_{50} polypeptides were purified by SDS/polyacrylamide-gel electrophoresis of immunoprecipitates obtained from radioiodinated D-positive red cells using human monoclonal anti-D. The samples were not reduced before electrophoresis. The D_{30} polypeptide was detected by its radioactivity, whereas the D_{50} polypeptide was detected by Coomassie Blue staining of samples run in parallel. Fig. 1(*c*) shows Coomassie Blue-stained gels of a typical immunoprecipitate. Amino acid analysis of the purified D_{30} polypeptide showed that it was obtained in a yield of 7.5% on the basis of the assumption that there are 30000 D-antigen sites per red cell (Hughes-Jones *et al.*, 1971). The *N*-terminal amino acid sequences of both the D_{30} and D_{50} polypeptides are shown in Fig. 2(*a*). The sequence of the D_{30} polypeptide was obtained in lower-than-expected yield (estimated to be 20%), but was obtained up to residue 32. The low yield suggests that the *N*-terminus of the D_{30} polypeptide is partially blocked. Two other groups have reported similar amino acid sequence studies on the D_{30} polypeptide and also obtained low sequencing yields; however, they only reported sequence up to residues 16 and 21 respectively (Bloy *et al.*, 1988; Saboori *et al.*, 1988). The sequence contains tyrosine residues at positions 4 and 28, but these do not contain any ^{125}I radioactivity, suggesting that they are not accessible to radioiodination by lactoperoxidase in the intact red cell. The *N*-terminus of the sequence from residues 1 to 10 has a polar character, suggesting that it is exposed at a membrane surface. The lack of radioactivity at tyrosine-4 and the presence of a cluster of basic residues at positions 3, 6, 9 and 10 suggest that this region of the protein is exposed to the cytoplasm. A segment of 19 hydrophobic residues follow residue 10, interrupted in the centre by glutamic acid-20. This region may represent a membrane-spanning region.

The *N*-terminal sequence of the D_{50} polypeptide was obtained in almost quantitative yield and is shown in Fig. 2(*a*). The sequence is clearly different from that of the D_{30} polypeptide. The *N*-terminal sequence up to valine-24 is comprised of hydrophobic amino acids, except for glutamic acid-13. The region to valine-24 is of sufficient length to span the membrane and is followed on the *C*-terminal side by a sequence containing polar amino acids which are likely to be located at a membrane surface.

N-Terminal sequence of the $R6A_{32}$ and $R6A_{45}$ polypeptides

Although these polypeptides were first defined by using monoclonal antibody R6A (Ridgwell *et al.*, 1983; Avent *et al.*, 1988), other monoclonal antibodies of the same specificity [BRIC 69 (Avent *et al.*, 1988) and BRIC 207 (see the Materials and methods section)] have subsequently been made which are more suitable for preparative immunoprecipitation. Fig. 1(*a*) shows the results of immunoprecipitation experiments from radioiodinated red cells using BRIC 69 (Fig. 1*e*) and BRIC 207 (Fig. 1*g*) and, on a preparative scale, from unlabelled red cells [BRIC 69 (Fig. 1*b*)]. The $R6A_{32}$ and $R6A_{45}$ polypeptides were purified after SDS/polyacrylamide-gel electrophoresis of immunoprecipitates obtained from red cells. The *N*-terminal sequences of the $R6A_{32}$ and $R6A_{45}$ polypeptides showed no differences from those of the D_{30} and D_{50} polypeptides respectively (Fig. 2*b*). Residue 2 of the $R6A_{45}$ polypeptide was shown to be cysteine by using the *S*-pyridylethylation method (Amons, 1987).

Although the D_{30} and $R6A_{32}$ polypeptides have the same *N*-terminal sequences, they are clearly different molecules. The differences in M_r between the two components has been shown to be due to the presence of an *N*-glycan on the $R6A_{32}$ polypeptide, since treatment of this polypeptide with an endoglycosidase F preparation decreases its M_r to that of the D_{30} polypeptide (Avent *et al.*, 1988). It is not clear whether this glycosylation difference is due to amino acid sequence differences between the polypeptides or a reflection of conformational factors which allow glycosylation of one polypeptide and not the other. The fact that D-negative red cells react with the R6A group of monoclonal antibodies (Anstee & Edwards, 1982) suggests a structural difference between the two polypeptides. Moore & Green (1987) have shown that antisera to the c and E antigen of the Rh system immunoprecipitate a component which is of similar molecular size to the $R6A_{32}$ polypeptide. It is possible that these antigens are carried on the $R6A_{32}$ polypeptide.

A similar close sequence relationship exists between the D_{50} polypeptide and the $R6A_{45}$ polypeptide. The two molecules clearly differ in molecular size and also differ in their ability to be radioiodinated (compare Figs. 1*e*

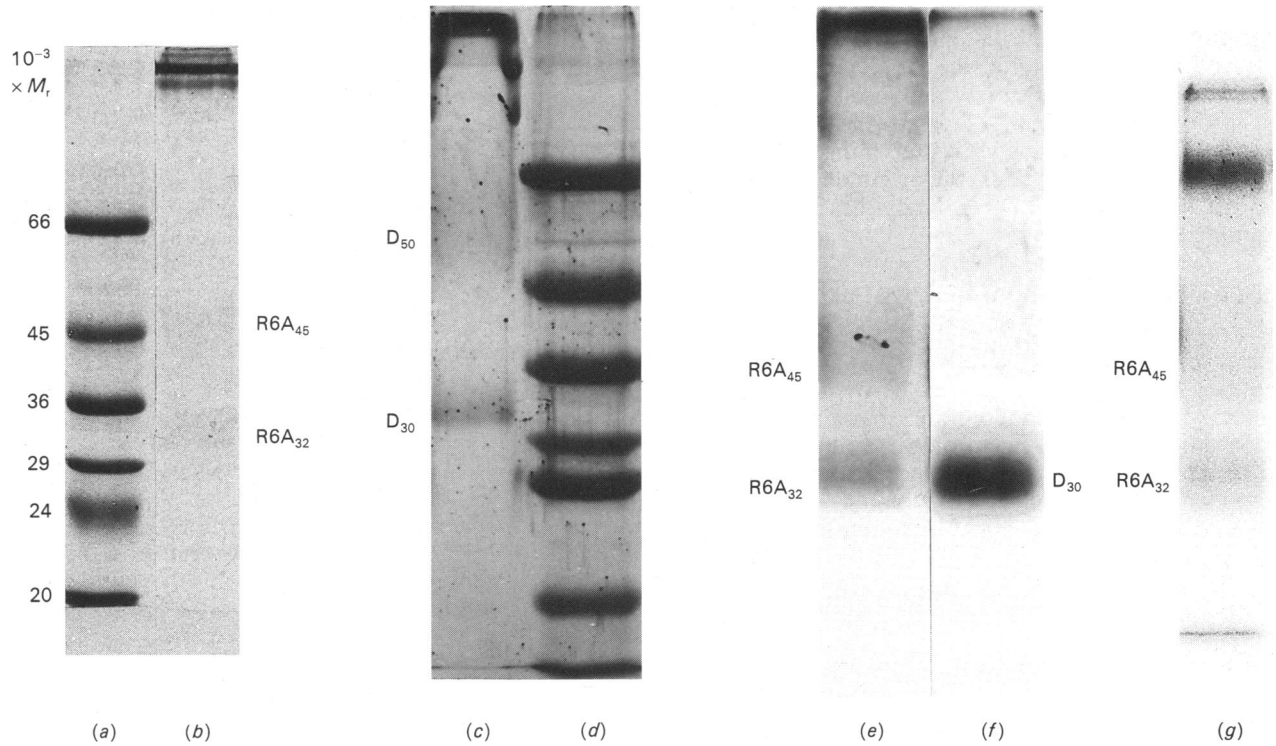

Fig. 1. SDS/polyacrylamide-gel electrophoresis of membrane components immunoprecipitated from intact erythrocytes

(*a–d*) Coomassie Blue-stained gels of: (*a*) Sigma SDS-7 molecular-size marker; (*b*) components immunoprecipitated by BRIC 69; (*c*) components immunoprecipitated by anti-Rh D; (*d*) Sigma SDS-7 molecular-size marker. (*e–g*) Autoradiographs of: (*e*) components immunoprecipitated by BRIC 69; (*f*) components immunoprecipitated by anti-Rh D; (*g*) components immunoprecipitated by BRIC 207. Tracks (*a*)+(*b*), (*e*)+(*f*) and (*g*) are from different gels.

(*a*)

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                    1       5       10      15      20      25      30      35
                    .       .       .       .       .       .       .       .
D30 polypeptide     S S K Y P R S V R R X L P L W A L T L E A A L I L L F Y F F T H
D50 polypeptide     M X F T F P L M A I V L E I A M I V L F G L F V E Y E T D Q T V L E Q L P X T
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(*b*)

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R6A32 polypeptide   S S K Y P R S V R R X L P
R6A45 polypeptide   M C F T F P L M A I V L E I A M I V L F G L F V E Y E T D Q
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(*c*)

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                    12                                  30
                    .                                   .
D30                 L P L W A L T L E A A L I L L F Y F F
                    . : :   : .   : :   : . : . : :   . :
D50, R6A45          F P L M A I V L E I A M I V L F G L F
                    .                                   .
                    5                                   23
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Fig. 2. *N*-Terminal amino acid sequences of Rh-related polypeptides

(*a*) *N*-Terminal amino acid sequences of D_{30} and D_{50} polypeptides. Residues which could not be assigned are indicated by X; (*b*) *N*-Terminal amino acid sequences of $R6A_{32}$ and $R6A_{45}$ polypeptides. The phenylthiohydantoin derivative of lysine was obtained as well as that of arginine at cycle 9 during the sequencing of the $R6A_{32}$ polypeptide. (*c*) Homology of the putative *N*-terminal membrane-spanning regions of the Rh-related polypeptides. Homology alignments were made by using the Wordsearch and Segments computer programs (University of Wisconsin Genetics Computer Group). Identical pairs of residues are indicated by ':'; pairs of residues marked by the computer program as evolutionarily homologous are indicated by '.'.

and 1*f*). The $R6A_{45}$ polypeptide is extensively *N*-glycosylated, since treatment with an endoglycosidase F preparation decreases the M_r of the molecule to 30000 (Avent *et al.*, 1988). The D_{50} polypeptide is also glycosylated (Moore & Green, 1987), but the M_r of the deglycosylated form has not been determined. As with the D_{30} and $R6A_{32}$ polypeptides, the origin of differences between the $R6A_{45}$ and the D_{50} polypeptides remains to be determined.

Sequence relationships between Rh-related polypeptides

Comparison of the *N*-terminal sequences of the D_{30} and $R6A_{32}$ polypeptides with that of the D_{50} and $R6A_{45}$ polypeptides suggests that they may be homologous in their putative *N*-terminal membrane-spanning regions (Fig. 2*c*). Homologies in membrane-spanning regions have to be treated with caution, because of the restricted set of amino acids found in these domains. However, search of the National Biomedical Research Foundation and the Swissprot protein sequence data bases with the putative membrane-spanning regions of either of the two groups of proteins examined here did not reveal any other sequence with the degree of homology which is apparent in Fig. 2(*c*). The relatedness of these proteins in their putative membrane-spanning regions provides a possible explanation for the association of D_{30} with D_{50}, and $R6A_{32}$ with $R6A_{45}$, during co-precipitation. One of the other components deficient in Rh_{null} cells, the BRIC 125 polypeptide (Avent *et al.*, 1988), was also purified by immunoprecipitation and gel electrophoresis. Three attempts to obtain an *N*-terminal sequence for this polypeptide failed, suggesting that it has a blocked *N*-terminus. This supports other evidence (Avent *et al.*, 1988) that the BRIC 125 polypeptide is distinct from the other polypeptides discussed here.

Concluding remarks

It has been assumed in previous studies that the D_{30} and $R6A_{32}$ polypeptides express the epitopes recognized by anti-D antibodies and monoclonal antibodies of the R6A group respectively. The results obtained by Moore & Green (1987) and those described here clearly indicate that each of these antibodies immunoprecipitate two different polypeptides. Whether or not these epitopes are present on one or both of the components, or are dependent upon the association of the two components, remains to be determined.

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