

cDNA cloning of a 30 kDa erythrocyte membrane protein associated with Rh (Rhesus)-blood-group-antigen expression

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The Rh-blood-group antigens (often described as Rhesus antigens) are associated with erythrocyte membrane proteins of approx. 30 kDa. We have determined the *N*-terminal 54 amino acid residues of the 30 kDa Rh D polypeptide (D₃₀ polypeptide). We used primers based on these sequence data and the polymerase chain reaction (PCR) on human reticulocyte cDNA and genomic DNA to clone two types of PCR product of identical size. The two PCR products had related translated amino acid sequences between the 3' ends of the primers, one of which was identical with that found for the D₃₀ polypeptide. We designate the two related mRNA species which gave rise to the PCR products as Rh30A and Rh30B, the latter corresponding to the D₃₀ polypeptide. We have isolated cDNA clones for the Rh30A protein which encode a hydrophobic membrane protein of 417 amino acids. The Rh30A protein has the same *N*-terminal 41 amino acids as the D₃₀ polypeptide, but beyond this point the sequence differs, but is clearly related. The Rh30A protein probably corresponds to the R6A₃₂ polypeptide, another member of the Rh 30 kDa family of proteins, which may carry the C/c and/or E/e antigens. Hydrophathy analysis suggests that the Rh30A protein has up to 12 transmembrane domains. Three of these domains are bordered by a novel cysteine-containing motif, which might signal substitutions at these cysteine residues. Information which supplements this paper (amino-acid-sequence-analysis histograms) is reported in Supplementary Publication SUP 50160 (4 pages), which has been deposited 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. (1990) 265, 5.

INTRODUCTION

The Rh-blood-group system (often described as Rhesus) is of considerable significance in immunohaematology and probably second in importance only to the ABH system. The system is comprised of numerous antigens, the most polymorphic of which are the antigens C, c, E, e and D. The Rh antigens are of particular clinical importance because of their involvement in haemolytic disease of the newborn (Mollison *et al.*, 1987). Despite their importance, the structure and sequence of the proteins carrying the antigens remain poorly characterized. It has been suggested that the Rh polypeptides may be involved in maintaining the asymmetric distribution of phospholipids in the erythrocyte membrane (Kuypers *et al.*, 1984; de Vetten & Agre, 1988; Connor & Schroit, 1989). Immunoprecipitation studies using human polyclonal and monoclonal anti-D have identified a 30 kDa membrane polypeptide (D₃₀ polypeptide) that appears not to be glycosylated (Moore *et al.*, 1982; Gahmberg, 1982, 1983). Anti-c and anti-E, as well as the murine monoclonal antibody R6A, immunoprecipitate related polypeptides (Moore *et al.*, 1982; Blanchard *et al.*, 1988; Moore & Green, 1987; Ridgwell *et al.*, 1983; Avent *et al.*, 1988a). Amino-acid-sequence analysis of the 32 kDa membrane component immunoprecipitated by R6A (R6A₃₂ polypeptide) showed that it had an *N*-terminal amino acid sequence that was identical with the *N*-terminal sequence of the D₃₀ polypeptide (Avent *et al.*, 1988b; Bloy *et al.*, 1988; Saboori *et al.*, 1988). We describe here the sequence of a cDNA for a member of the D₃₀/R6A₃₂ group of proteins, which we denote the Rh30A protein. The cDNA probably corresponds

to the R6A₃₂ polypeptide and may carry the C/c and/or E/e antigens. We also report the presence of an mRNA in erythroid cells for a second member of this group of proteins, the partial amino acid sequence of which corresponds with the D₃₀ polypeptide. Information which supplements this paper (amino-acid-sequence-analysis histograms) is reported in Supplementary Publication SUP 50160 (4 pages), which has been deposited 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. (1990) 265, 5.

MATERIALS AND METHODS

Purification and *N*-terminal sequencing of the D30 polypeptide

D₃₀ polypeptide was purified and sequenced as described previously (Avent *et al.*, 1988b) from 500 ml of outdated human whole blood (presumptive Rh genotype cDE/cDE) using 1500 ml of anti-D culture supernatant. Approx. 7 nmol of D₃₀ polypeptide was purified, of which approx. 1 nmol was loaded on to a pulsed liquid-phase protein sequencer (model 477A; Applied Biosystems International).

Polymerase-chain-reaction (PCR) amplification and cloning and sequencing of PCR products

PCR was done with human reticulocyte cDNA templates [prepared as described by Temple *et al.* (1977) and Maniatis *et al.* (1982)] using *Taq* polymerase (Perkin-Elmer/Cetus) according to

Abbreviations used: PCR, polymerase chain reaction; nt, nucleotide.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide sequence Databases under the accession number X54534.

RESULTS AND DISCUSSION

Cloning of PCR amplification products corresponding to 30 kDa Rh proteins

We were unsuccessful in obtaining cDNA clones for the 30 kDa Rh proteins using probes based on the previously published amino-acid-sequence data for the D₃₀ and R6A₃₂ polypeptides (*N*-terminal sequence up to residue 32; Avent *et al.*, 1988b). We therefore determined further *N*-terminal amino acid sequence for the D₃₀ polypeptide up to residue 54 (Fig. 1a). The R6A₃₂ polypeptide gave an identical amino acid sequence up to residue 41, but we were unable to obtain sequence data beyond this point. Mixed sequence oligonucleotides based on the D₃₀ polypeptide amino acid sequence were prepared. By using PCR with these oligonucleotide primers and reticulocyte cDNA, PCR products of the predicted size (88 bp) were obtained (Fig. 1b). After cloning and sequencing, the PCR products were found to be of two types of identical size. The translated amino acid sequence between the 3' ends of the primers of one type (Rh30PCR-B) was found to be identical with that of the D₃₀ polypeptide amino acid sequence (residues 36–46; Fig. 1b). The sequence between the 3' ends of the primers of the other type (Rh30PCR-A) was clearly related to that of Rh30PCR-B and showed conservation of eight of the 11 amino acid residues coded for in this portion of the sequence (Fig. 1b) and 60% DNA sequence similarity. This shows that at least two related mRNA species of the D₃₀/R6A₃₂ polypeptide family are present in erythroid cells. We designate the mRNAs which gave rise to the Rh30PCR-A and Rh30PCR-B products as Rh30A and Rh30B respectively.

Isolation and sequence of RH30A cDNA clones

The 88 bp products of PCR (containing both Rh30PCR-A and Rh30PCR-B) were used to screen a human bone-marrow cDNA library. Two positive cDNA clones of different size were isolated with the same DNA sequence where they overlapped. The translated amino acid sequence of the cDNA yielded the *N*-terminal amino acid sequence of the D₃₀/R6A₃₂ polypeptides up to residue 41 (amino acids 2–42 in Fig. 2). The DNA sequence of the clones corresponding to amino acid residues 37–47; Fig. 2) also perfectly matched the nucleotide sequence between the primers of the PCR product Rh30PCR-A (Fig. 1b), showing that the cDNA clones correspond to the Rh30A mRNA. The Rh30A cDNA predicted an open reading frame of 417 amino acids starting from the Met codon at nucleotide (nt) 1 (Fig. 2). This Met codon is preceded by a purine residue at position –3, which is thought to be important in translation initiation (Kozak, 1986) and is likely to be the initiator Met codon. A Met codon is also present at nt –43 in a different reading frame upstream of this, but this is followed by an in-frame termination codon at nt 2. *N*-Terminal amino acid sequencing of the D₃₀/R6A₃₂ polypeptides (Avent *et al.*, 1988b; Bloy *et al.*, 1988; Saboori *et al.*, 1988) has shown that the sequences of the mature proteins start at Ser-2, so the initiating methionine residue is post-translationally cleaved. The composite 1466-bp cDNA contains 44 nts of 5' non-coding region and 171 nts of 3' non-coding region (Fig. 2). The 3' end of the cDNA terminated with an A₁₆ tract, but no sequence matching the consensus polyadenylation signal AATAAA was present close upstream of the A₁₆ tract. It is possible that the 3' end of the mRNA extends further than the 3' end of our clones.

Structure of Rh30A protein

Hydropathy analysis of the predicted Rh30A sequence using a 20-amino-acid window showed that it contained two extended hydrophobic regions of sequence as well as five hydrophobic

sections with the lengths expected for single membrane-spanning segments (Fig. 3a). The overall hydrophobic nature of the protein is apparent from the hydropathy plot and consistent with the properties of the D₃₀/R6A₃₂ polypeptide (de Vetten & Agre, 1988). The two long hydrophobic sections of sequence comprise residues 76–153 (77 amino acids) and 273–388 (115 amino acids). Examination of the sequence shows the presence of small clusters of polar amino acids within these long hydrophobic sections, which become evident as troughs in the hydropathy profile when the analysis is done using a smaller window of nine amino acids (Fig. 3b), but are obscured because of their short length when the analysis is done using the wider window. These polar clusters probably represent regions of polypeptide at the membrane surface and the first extended hydrophobic region could span the membrane three times, whereas the second could span the membrane four times. Overall the protein probably contains twelve membrane-spanning domains. The *N*- and *C*-terminal halves of the protein show similarities in their hydropathy profiles, suggesting that the protein may comprise two domains with similar structure, each containing six membrane-spanning regions. Analysis of the sequence using the DIAGON method (Staden, 1982) did not, however, yield any evidence for the two halves of the protein having similar amino acid sequences. However, the related sequences WKAPHVA and WKFPHLA are found adjacent to each other in the *C*-terminal tail of the protein and a 13-nt DNA sequence in the 5' non-coding sequence (nts –38 to –26) is directly repeated in the coding region (nts 425–437). Four acidic residues (Glu-21, Asp-95, Glu-156 and Glu-340) and one basic residue (Arg-114) are present in putative membrane-spanning sequences and could be located within the membrane interior (Fig. 3c).

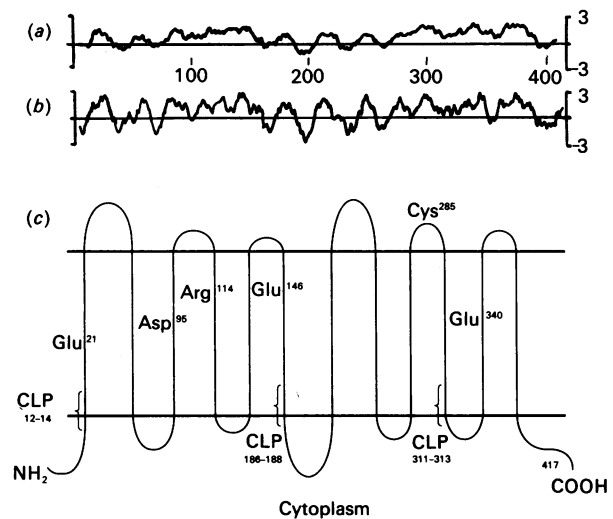


Fig. 3. Hydropathy analysis and schematic diagram of the possible topology of the Rh30A protein

(a) Kyte–Doolittle hydropathy analysis using a window of 20 amino acids. (b) Kyte–Doolittle hydropathy analysis using a window of nine amino acids. Hydrophobicity is shown as the vertical axis with the hydrophobic side of the plot having a positive value. The horizontal axis shows the amino acid residue number along the sequence. Analysis was done using the program PEPLOT in the University of Wisconsin Genetics Computer Group package (Devereux *et al.*, 1984). (c) Possible topology of the Rh30A protein. This schematic diagram assumes the presence of 12 membrane-spanning segments in the protein and shows the possible locations of the CLP motifs, the extracellular cysteine residue and the acidic and basic residues which appear to be located within the membrane interior.

The lack of a cleaved *N*-terminal hydrophobic signal sequence suggests that the first membrane-spanning domain of the Rh30A protein acts as an uncleaved signal-anchor sequence. The sequences adjacent to this first membrane span fit the criteria described for signal-anchor sequences (Hartmann *et al.*, 1989; Dalbey, 1990; von Heijne, 1990). The sequence on the *N*-terminal side of the hydrophobic region is strongly basic [+5 using the criteria of Hartmann *et al.* (1989)], whereas that in the 15 amino acids flanking the *C*-terminal side of the hydrophobic region is predominantly acidic (-1.5). The charge difference between the flanking regions of +6.5 charges towards the *N*-terminus strongly predicts that the *N*-terminus is located on the cytoplasmic side of the membrane (Hartmann *et al.*, 1989). A schematic diagram of the possible topology of the protein based on this and the hydrophobic analysis is shown in Fig. 3(c). Treatment of erythrocytes with thiol reagents abolishes Rh C and D antigen activity (Green, 1965, 1967). An extracellular thiol group is necessary for D antigen activity, since an impermeant maleimide (glutathione maleimide) inactivates the D antigen activity of intact erythrocytes (Abbott & Schachter, 1976). The model in Fig. 3(c) suggests that only one cysteine residue (Cys-285) is extracellular, and this may be the thiol required for Rh antigen activity.

Three of the six cysteine residues in the protein (Cys-12, Cys-186 and Cys-311 in Fig. 2) form part of the sequence motif CLP, and these are found at locations close to the points at where the polypeptide chain enters the membrane. A basic residue is located immediately adjacent to the *N*- or *C*-terminal side of the CLP motif, and another basic amino acid is present five residues away from the *N*- or *C*-terminal side of the motif in regions likely to be present at the membrane surface (Fig. 3c). The 30 kDa Rh polypeptides have been shown to be the major palmitoylated components of the erythrocyte membrane (Staufenbiel, 1988; de Vetten & Agre, 1988), and de Vetten & Agre (1988) suggest that each protein molecule is acylated several times with palmitoyl residues. Direct protein sequencing of the 30 kDa Rh polypeptides did not yield an identifiable residue at the position corresponding to Cys-12 (the cysteine in the first CLP motif), even after pyridylethylation of the polypeptide (Avent *et al.*, 1988b), suggesting that this cysteine residue is substituted in some way, and it is possible that it is palmitoylated. The other cysteine residues located in CLP motifs may be similarly substituted. However, it is noteworthy that, in another polytopic membrane protein, namely bovine rhodopsin, the two cysteine residues in the sequence CCGK (residues 323-324) are both palmitoylated (Ovchinnikov *et al.*, 1988). This sequence occurs at the cytoplasmic surface of the membrane on the *C*-terminal side of a membrane-spanning domain. A similar sequence CCNR occurs in the Rh30A protein (residues 315-318), and is also likely to be at the membrane surface. These cysteine residues could alternatively be the sites of palmitoylation. The asymmetric distribution of phospholipids in the erythrocyte membrane results from an ATP-dependent transport system (Seigneuret & Devaux, 1984), and it has been suggested that the 30 kDa Rh polypeptides are involved in this process (Kuypers *et al.*, 1984; de Vetten & Agre, 1988; Connor & Schroit, 1989). The CLP motifs in the Rh30A protein may have a functional role in this process.

Relationship of Rh30A protein to the Rh antigens

The 41 residues of the *N*-terminal amino acid sequence of the R6A₃₂ polypeptide which we have been able to establish by protein sequencing are identical with that predicted from the Rh30A cDNA (residues 2-42). This portion of the sequence is also the same as the D₃₀ polypeptide sequence (Fig. 1a). Immediately adjacent to this sequence are the three amino-acid-sequence differences which are so far known to distinguish the

D₃₀ from the Rh30A protein. The Rh30A protein therefore corresponds to the R6A₃₂ polypeptide rather than the D₃₀ polypeptide. There are no potential *N*-glycosylation sites (NXS/T) in the predicted Rh30A protein sequence. It has been shown that the D₃₀ polypeptide is not glycosylated (Gahmberg, 1983; Avent *et al.*, 1988a), and it is likely that the Rh30A protein is similar in this respect. We previously suggested that the R6A₃₂ polypeptide was *N*-glycosylated, on the basis of a shift in *M_r* of the purified protein on treatment with endoglycosidase preparations (Avent *et al.*, 1988a). A likely explanation for this observation is that it was due to cleavage resulting from proteinase contamination of the partially purified endoglycosidase mixture used in these experiments.

We have shown that mRNAs for two members of the D₃₀/R6A₃₂ polypeptide family are present in erythroid cells. The portion of the Rh30B mRNA cloned in the Rh30PCR-B clone (Fig. 1b) corresponds in translated amino acid sequence to that found for the D₃₀ polypeptide (Fig. 1a). It is possible that other members of this family are also present in erythrocytes, and it has been suggested that the C and E antigens are present on different polypeptides (Blanchard *et al.*, 1988). Although the Rh30A polypeptide does not give rise to the Rh D antigen, the C/c and/or E/e antigens may arise from polymorphic structures in this protein. The availability of cDNA clones for members of this family should provide a basis for elucidating the structures responsible for determining the different antigens of the Rh system.

Note added in proof (received 10 September 1990)

After the submission of this paper a report appeared describing the isolation of a cDNA clone which is the same as the Rh30A cDNA (Cherif-Zahar *et al.*, 1990).

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