The Rh blood group antigens are associated with non-glycosylated human erythrocyte membrane proteins of molecular mass 30 kDa (the Rh50A polypeptides) and a glycoprotein of 40–100 kDa (the Rh glycoprotein). We have studied the topology of this family of proteins in the erythrocyte membrane. We confirmed the predicted cytosolic localization of the C and N termini of the Rh protein family. We located Lys-196 and Arg-323 of the Rh glycoprotein to the cytosol, and Glu-34 to the extracellular side of the plasma membrane in erythrocytes, by N-terminal sequencing of Rh glycoprotein peptides produced by proteolysis at the cytoplasmic or extracellular side of the membrane. We also show that a glycine chain is present on only one (Asn-37) of the three potential N-glycan addition sites in the Rh glycoprotein. Studies of the Rh glycoprotein fragments that co-immunoprecipitated with the Rh50A polypeptides suggest there is an interaction between the Rh50A polypeptides and amino acids 35–196 of the Rh glycoprotein. A model for the organization of the components of the Rh complex in the red cell membrane is proposed.

The antigens of the human Rh (Rhesus) blood group system are important in transfusion medicine and immunohematology because of their immunogenicity in man and involvement in hemolytic disease of the newborn (reviewed by Race and Sanger, 1968) and Hadley and Kumpel (1993)). Some of the erythrocyte membrane proteins that carry Rh antigens have been characterized by immunoprecipitation studies using anti-Rh/C/c, anti-Rh/D, or anti-Rh/E/e antisera and Rh-related monoclonal antibodies of the R6A type (reviewed by Agre and Cartron, 1991 and Anstee and Tanner, 1993)). Two Rh-related polypeptides of 30 and 32 kDa, the Rh50A polypeptides, have been identified and these are unusual in that they are not glycosylated (Moore et al., 1982; Gahmberg et al., 1982; Ridgwell et al., 1983; Moore and Green, 1987). The N-terminal protein sequences of both these polypeptides have been determined (Avent et al., 1988b; Bloy et al., 1988) and cDNA clones encoding members of this group of polypeptides have been isolated. Two cDNA clones, Rh30A (RhXb) and Rh13, predict hydrophobic membrane proteins of 417 amino acids which are very similar in amino acid sequence (Avent et al., 1990; Cherif-Zahar et al., 1990; Le Van Kim et al., 1992b; Kaji and Ikemoto, 1992; Kaji et al., 1993). Hydropathy analysis of the cDNA clones suggests that the proteins contain 12 or 13 membrane spanning domains (Avent et al., 1990; Cherif-Zahar et al., 1990). It has been suggested that the C termini of the proteins are located on the extracellular side of the plasma membrane (Krahmer and Prohaska, 1987; Bloy et al., 1990; Cherif-Zahar et al., 1990) consistent with the proteins having an odd number of up to 13 membrane spanning regions. However, other evidence (Avent et al., 1992) suggests that the C termini are on the cytoplasmic side of the plasma membrane, and that the proteins have an even number of up to 12 membrane spanning regions.

Studies with Rh-specific monoclonal antibodies have also identified Rh-related glycoproteins that co-precipitate with the Rh50A polypeptides and migrate as a diffuse band on SDS-PAGE (Moore and Green, 1987; Avent et al., 1988b). The available evidence suggests that there is only one Rh glycoprotein polypeptide (Ridgwell et al., 1994). Recently a cDNA clone (Rh50A) was isolated for the Rh50A glycoprotein (Ridgwell et al., 1992). The Rh50A cDNA clone predicts a polypeptide of 409 amino acids with three potential N-glycosylation sites, two of which are predicted to be extracellular. The Rh50A glycoprotein amino acid sequence is clearly homologous to that of the Rh30A polypeptide, and the Rh30A and Rh50A proteins probably both have the same topology, and belong to a family of structurally related membrane proteins (Ridgwell et al., 1992).

Since there is disagreement about the topology of the Rh-related polypeptides, we have further investigated this question with different members of the Rh protein family. We have studied the location of their C and N termini using polyclonal antibodies reactive with the N- and C-terminal amino acid sequences of the Rh50A protein and the C terminus of the Rh50A protein. Although the Rh50A cDNA sequence contains three potential N-glycan addition sites, we show that the Rh glycoprotein contains a single N-glycan located in the first extracellular loop of the protein. We have also determined the location of proteolytic cleavage sites in the amino acid sequence of the native Rh glycoprotein in the erythrocyte membrane. This data provides experimental evidence for the topological location of several sites in the amino acid sequence of the Rh glycoprotein, and by homology, the Rh50A polypeptide family.

MATERIALS AND METHODS

Rh-specific Polyclonal Antibodies and Monoclonal Antibodies—The polyclonal antibodies reactive with the C and N termini of the Rh30A protein and the C terminus of the Rh50A glycoprotein have been described elsewhere (Ridgwell et al., 1994). Rh30A anti-C was raised to an 18-amino acid peptide corresponding to the C-terminal residues 401–417 of the predicted Rh30A polypeptide. Rh30A anti-N was raised to an 11-amino acid peptide corresponding to the N-terminal residues 2–12 of the predicted Rh30A polypeptide. RhGP anti-C was raised to a 14-amino acid peptide that corresponds to the C-terminal residues 396–409 of the predicted Rh50A glycoprotein sequence. Mouse monoclonal antibodies BRIC 69 and BRIC 207 are of the R6A type and react with the Rh proteins irrespective of the Rh phenotype of the red cells. BRIC 69, BRIC 207, and the human monoclonal anti-Rh(D) antibody (AB5) were provided by Dr. D. J. Anstee, International Blood Group Reference
Immunoprecipitation from Intact Erythrocytes and Erythrocyte Membranes—Rh30 polypeptides and Rh glycoproteins were immunoprecipitated from intact erythrocytes as described by Moore et al. (1982) except that KI washing steps were included as follows to reduce nonspecific background (Bennett, 1983). Prior to overnight incubation with antibody at 4 °C, intact erythrocytes were washed twice with phosphate-buffered saline (PBS), with 10 volumes of ice-cold 1 × KI, 5 mM sodium phosphate, pH 8.0 (KI/P8), and finally with ice-cold PBS until no lysis was observed. Erythrocyte membranes were prepared as described by Dodge et al. (1983) and washed once with ice-cold KI/P8, then with an excess of 5 mM sodium phosphate, pH 8.0 (P8), and incubated overnight at 4 °C with the antibody. Membranes containing bound antibody were then washed twice with P8, twice with KI/P8, and two more times with P8 prior to the addition of protein A-Sepharose (Bioprocessing Ltd.). When the Rh glycoprotein was immunoprecipitated from PNGase F digested intact erythrocytes or erythrocyte membranes from PNGase F-treated erythrocytes, the intact erythrocytes and erythrocyte membranes were not washed with KI. For large-scale immunoprecipitations from erythrocyte membranes using the Rh-specific antipeptide polyclonal antibodies, 10 mg BSA and 0.1% (v/v) digoxin were added to the antibody incubation mixture, and all buffers contained 1 mM EDTA and 0.1% digitonin to prevent resealing of the membranes. Immunoprecipitates were analyzed using the Tricine buffer SDS-PAGE system of Schagger (1987).

Proteolytic Digestion of Intact Erythrocytes and Erythrocyte Membranes—Erythrocytes were washed in PBS and membranes were prepared by lysis in P8 (Dodge et al., 1983). For trypsin digestion intact erythrocytes or erythrocyte membranes were washed and resuspended in 10 volumes of Tris, pH 7.4, 150 mM KCl containing 0.1 mg/ml tosylphenylalanine choloromethyl ketone-treated trypsin (Sigma), and incubated at 37 °C for 45 min. For SDS-PAGE analysis, sample buffer (Laemmli, 1970) containing protease inhibitors (5 μg/ml tosyllyl chloromethyl ketone, 0.5 μg phenylmethysulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor (Sigma)) was added to the digest. For immunoprecipitation from trypsin-treated erythrocytes or trypsin-treated erythrocyte membranes, the proteolysis reaction was stopped by the addition of the above protease inhibitors, and the digested material was washed several times with PBS (in the case of intact erythrocytes) or P8 (in the case of erythrocyte membranes). For digestion with chymotrypsin, intact erythrocytes or erythrocyte membranes were washed with 10 volumes of Tris, 250 mM NaCl, resuspended in the same buffer containing 2.5 mg/ml chymotrypsin (Sigma), and incubated for 45 min at 37 °C. The reaction was stopped with the protease inhibitors phenylmethylsulfonyl fluoride (0.5 μM) and chymostatin (0.1 mg/ml). Staphylococcus aureus V8 protease (0.5 mg/ml; Sigma) digestions were carried out under similar conditions as the chymotrypsin digestions except that the reaction was stopped using 125 μg/ml o-mercaptoethanol (Boehringer Mannheim) and 0.5 μM phenylmethysulfonyl fluoride. Digested intact erythrocytes or erythrocyte membranes were immunoprecipitated without KI washing as described above.

Immunoblotting—Immunoblotting was performed as described by Mallinson et al. (1986) except that samples were separated using the SDS-PAGE Tricine buffer system of Schägger & von Jagow (1987). Immobilon (Millipore) was used as the transfer membrane and 5% (v/v) bovine dried skimmed milk powder in PBS, 0.2% (v/v) Tween 20 (Sigma) was used as blocking agent.

Immunoprecipitation and N-terminal Sequencing of Rh Glycoprotein Proteolytic Peptides—2.5 ml of washed packed erythrocytes were deglycosylated using PNGase F, digested with S. aureus V8 protease, and immunoprecipitated without KI washing, as described above, using 10 ml of culture supernatant of BRIC 69 (Avent et al., 1986a). The BRIC 69 immunoprecipitate was solubilized from intact A-Sepharose beads using 400 μl of gel sample buffer (Laemmli, 1970) from which glycerol and β-mercaptoethanol had been omitted, and concentrated using a 10-kDa cut-off Durapore concentrator (Millipore). 5% glycerol was added to the concentrated solubilized immunoprecipitate, and proteins were separated on a 12% SDS-PAGE Tricine gel.

The Rh glycoprotein tryptic peptides were isolated from 3 ml of trypsin-digested erythrocyte membranes by immunoprecipitation using 7 ml of RhGP anti-C polyclonal rabbit antiserum. To purify the peptides for N-terminal amino acid sequencing, the immunoprecipitated peptides were separated by SDS-PAGE as described above, except that no β-mercaptoethanol was added to the gel sample buffer. The peptides in the gel were transferred onto Problott membrane (Applied Biosystems) and stained with Serva Blue G (Serva) as described by the membrane suppliers. The stained peptides were sequenced using a Blott cartridge and a modified Fastblott cycle (Ridgwell et al., 1994).

RESULTS

Immunoprecipitation of Rh Proteins with Rabbit Polyclonal Antibodies—Three Rh-specific antipeptide rabbit polyclonal antibodies were used in this work. Rh30A anti-C is a polyclonal antibody that was raised against the C-terminal 18 amino acid residues of the Rh30A polypeptide; Rh30A anti-N was raised against residues 2–12 at the N terminus of Rh30A; and RhGP anti-C was raised against the C-terminal 14 amino acids of the Rh50A glycoprotein. These antibodies detect the Rh30 polypeptides and Rh glycoproteins on immunoblots of red cell membranes irrespective of the Rh Cc, D, Ee phenotypes of the cells (Ridgwell et al., 1994). The antibodies were used to study the cellular location of the N and C termini of the Rh30 polypeptides and the C termini of the Rh glycoprotein by comparing their ability to immunoprecipitate the Rh polypeptides and Rh glycoproteins from intact erythrocytes and leaky erythrocyte membranes. Erythrocyte membranes were stripped of peripheral membrane proteins with KI prior to incubation with the antibodies. In order to ensure that this treatment did not uncover any extra-cellular antibody epitopes, the intact erythrocytes were also treated with KI before incubation with the antibodies.

The polyclonal antibodies Rh30A anti-N and RhGP anti-C were added to KI washed intact RhD-ve erythrocytes and the resulting immunoprecipitates were immunoblotted with the same antibodies. Neither Rh30A anti-N nor RhGP anti-C immunoprecipitated the Rh polypeptides from the KI-washed intact erythrocytes (Fig. 1, b and e). However, immunoblots of the immunoprecipitates from RhD-ve KI-washed intact erythrocytes demonstrated the presence of the 39–32-kDa Rh30 polypeptides in the Rh30A anti-N immunoprecipitate and the diffusely migrating Rh glycoprotein in the RhGP anti-C immunoprecipitate (Fig. 1, a and f). In similar experiments (data not shown), Rh30A anti-C immunoprecipitated the Rh30 polypeptides from KI-washed leaky red cell membranes but not from KI-washed intact erythrocytes, consistent with the results described by Avent et al. (1992).

No Rh glycoproteins were immunoprecipitated from PNGase F-treated intact erythrocytes by RhGP anti-C (Fig. 1). However, a 30.5-kDa band was immunoprecipitated by RhGP anti-C from membranes prepared from intact erythrocytes that had been deglycosylated with PNGase F (Fig. 1m). This band represents the deglycosylated Rh glycoprotein (Fig. 1i). For each of the above immunoprecipitation experiments a parallel control immunoprecipitation was carried out with the preimmune rabbit serum appropriate to each antibody. None of the preimmune sera immunoprecipitated Rh-specific bands (Fig. 1). These immunoprecipitation experiments demonstrate that the epitopes reactive with Rh30A anti-C, Rh30A anti-N, and RhGP anti-C are only accessible to the antibodies in leaky red cell membranes and not in intact erythrocytes. We conclude that both the C and N termini of the Rh family of polypeptides are located on the cytoplasmic side of the red cell membrane.

Protease Cleavage of Rh Glycoprotein in Intact Erythrocytes—We investigated the susceptibility of the Rh glycoprotein to proteolysis in intact erythrocytes in order to obtain further information on its topology within the membrane. In—
tact erythrocytes were deglycosylated with PNGase F, treated with S. aureus V8 protease, and then immunoprecipitated with the anti-Rh(D) monoclonal antibody AB5 (Avent et al., 1988a). The immunoprecipitated proteolytic fragments were separated by SDS-PAGE and immunoblotted with Rhgp anti-C. When intact glycosylated erythrocytes were digested with S. aureus V8 protease (Fig. 2), the Ms of the diffuse band corresponding to the Rh glycoprotein showed a slight shift to a lower Mr (Fig. 2, c and d). When intact erythrocytes were deglycosylated with PNGase F prior to treatment with S. aureus V8 protease, the apparent molecular mass of the band detected with Rhgp anti-C clearly shifted from 30.5 kDa (intact deglycosylated protein) to 28.5 kDa (Fig. 2, a and b).

These results show that the Rh glycoprotein in red blood cells contains an S. aureus V8 protease cleavage site which is extracellular. The S. aureus V8 protease fragment (RhGP.V8) extends from the proteolytic cleavage site to the C terminus of the Rh glycoprotein and can be immunoprecipitated with the anti-Rh(D)-specific monoclonal antibody AB5 and is also N-glycosylated. When intact erythrocytes were treated with trypsin (Fig. 2, e and f), the Rh glycoprotein did not show a detectable shift in Mr, suggesting that it contains no extracellular trypsin cleavage sites.

Protease Treatment of the Rh Glycoprotein in Leaky Erythrocyte Membranes—The susceptibility of the Rh glycoprotein to trypsin cleavage in leaky erythrocyte membranes was investigated. Untreated membranes or membranes from PNGase F-treated erythrocytes were digested with trypsin. The resulting membrane protein fragments were separated by SDS-PAGE and immunoblotted with Rhgp anti-C. Trypsin digestion of leaky erythrocyte membranes gave rise to at least 9 trypsic fragments that reacted with Rhgp anti-C (Fig. 2, g and i), and which therefore extend all the way to the C terminus of the Rh glycoprotein. PNGase F treatment of the erythrocytes prior to trypsin-treatment of the membranes did not affect the pattern of Rhgp anti-C reactive trypsin fragments on immunoblots (Fig. 2k). In particular, there was no difference in the mobility of the 21.5-kDa trypsin fragment (RhGP.T1) and the 7–7.5-kDa trypsin fragment (RhGP.T4), whether the erythrocytes had been deglycosylated with PNGase F or not, showing that they do not carry N-glycan chains. Although the same trypsic peptides were observed from membranes of erythrocytes of Rh phenotype CCD.e, cCD.E, or cede, there were differences in the amounts of each of the trypsin fragments (notably RhGP.T1) that reacted with Rhgp anti-C in trypsin digests of membranes from cells of different Rh phenotypes (Fig. 2, m–o), when low concentrations of the protease were used. These differences were reproduced in three different experiments. These results suggest that there is a difference in the accessibility of the cytoplasmic portion of the Rh glycoprotein to trypsin, which is dependent on Rh phenotype.

The 28.5-kDa S. aureus V8 protease fragment (RhGP.V8) of the Rh glycoprotein was precipitated by the anti-Rh(D) monoclonal antibody AB5 (Fig. 3, a–d), and by the Rh-specific monoclonal antibodies of the R6A type, BRIC 69 and BRIC 207 (data not shown; Avent et al., 1988a). This suggests that the RhGP.V8 peptide is either itself part of the antibody epitope or it interacts with the proteins carrying the Rh antibody epitopes. To further investigate this question, we used BRIC 69 and BRIC 207 to immunoprecipitate proteins from trypsin-treated erythrocyte membranes, and then used Rh30A anti-N, Rh30A anti-C, and Rhgp anti-C to investigate the presence of Rh30 polypeptide and Rh glycoprotein fragments in immunoblots of the immunoprecipitates. No trypsin cleavage products reactive with Rhgp anti-C were detected in immunoblots of the AB5, BRIC 69, or BRIC 207 immunoprecipitates (Fig. 3, a–c), although the expected C-terminal trypsinic fragments of the Rh glycoprotein were clearly present when the immunoprecipitation from the membranes was done using the Rhgp anti-C polyclonal antibody (Fig. 3d). However, trypsin cleavage products reactive with Rh30A anti-N and anti-C were detected in immunoblots of the BRIC 69 and BRIC 207 monoclonal antibody immunoprecipitates. This shows that these monoclonal antibodies precipitate the C-terminal and N-terminal trypsinic peptides derived from the Rh30 polypeptides but not the C-terminal fragments of the Rh glycoprotein. Therefore, unlike the RhGP.V8 fragment, the C-terminal trypsinic fragments of the Rh glycoprotein do not remain associated with the proteins that carry the Rh(D), BRIC 69, and BRIC 207 antibody epitopes in the red cell membrane.

N-terminal Amino Acid Sequencing of Rh Glycoprotein Proteolytic Peptides—In order to identify the location of the extracellular S. aureus V8 protease and cytoplasmic trypsin cleavage sites in the Rh glycoprotein amino acid sequence, the proteolytic fragments RhGP.V8, RhGP.T1, and RhGP.T4 were isolated by immunoprecipitation, separated by SDS-PAGE, and their N-terminal amino acid sequences determined. The N-terminal amino acid sequence obtained from the 28.5-kDa RhGP.V8 fragment, isolated from PNGase F-deglycosylated CCD.e erythrocytes using the monoclonal antibody BRIC 69, corresponded to amino acids 35–49 predicted by the Rh50A cDNA clone (Ridgwell et al., 1992), except that aspartic acid was observed in the RhGP.V8 peptide sequence at the residue where the Rh50A cDNA predicts asparagine (amino acid 37).
The 21.5-kDa RhGP.T1 and 7–7.5-kDa RhGP.T4 fragments isolated by immunoprecipitation from trypsin-treated erythrocyte membranes (cDDee phenotype) using the RhGP anti-C polyclonal antibody were also sequenced. The tryptic peptide RhGP.T1 corresponded to amino acids 197–206 of the predicted Rh50A amino acid sequence, and the N-terminal sequence of RhGP.T4 corresponded to amino acids 224–334 of the Rh50A cDNA sequence. These results locate the S. aureus V8 protease cleavage site at Glu-34 of the Rh glycoprotein to the extracellular face of the erythrocyte membrane, and the two trypsin cleavage sites at Lys-196 and Arg-523 to the cytoplasmic surface of the membrane.

DISCUSSION

The N and C Termini of the Rh Protein Family Are Located in the Red Cell Cytoplasm—Hydropathy analysis and amino acid sequence alignment of Rh30A and Rh50A suggests that the Rh30 polypeptides and Rh glycoproteins have a similar topology in the erythrocyte membrane (Avent et al., 1990; Ridgwell et al., 1992). Two different membrane orientations have been proposed for the Rh30 polypeptides. Both predict a cytosolic N terminus, but one report suggests that the C terminus of the Rh30 polypeptide is located in the red cell cytoplasm (Avent et al., 1992), while other workers have suggested it is at the extracellular face of the red cell membrane (Bloy et al., 1990; Cherif-Zahar et al., 1990). The cytosolic location of the N termini of the Rh family of polypeptides is predicted by the absence of cleaved leader sequences and the charge distribution flanking the first membrane spanning segment (Avent et al., 1990; Cherif-Zahar et al., 1990; Ridgwell et al., 1992). Our immunoprecipitation studies with a rabbit polyclonal antibody raised against the N-terminal 12 amino acids of the Rh30A polypeptide (Rh30A anti-N) provide experimental evidence for the cytosolic location of the N terminus of this family of proteins. The Rh30 polypeptide was immunoprecipitated by Rh30A anti-N from leaky erythrocyte membranes but not from intact erythrocytes.

Both the Rh30 polypeptides and Rh glycoproteins could be immunoprecipitated from leaky erythrocyte membranes, but not from intact erythrocytes, by antibodies reactive against the C termini of the Rh30 polypeptide and the Rh glycoprotein (Rh30A anti-C and RhGP anti-C, respectively). The removal of bulky glycans from the erythrocyte surface by PNGase F treatment did not uncover the RhGP anti-C epitope in intact erythrocytes: the Rh glycoproteins could only be immunoprecipitated from leaky erythrocyte membranes, whereas the red cells were deglycosylated or not. These results suggest that the C terminus of both the Rh30A polypeptide and the Rh glycoprotein is located in the cytoplasm. This is further demonstrated by the susceptibility of the Rh30A anti-C antibody epitope to carboxypeptidase Y digestion. This epitope was not destroyed by carboxypeptidase Y digestion of intact erythrocytes, but was lost after endopeptidase digestion of leaky membrane preparations (Avent et al., 1992).

A Single Site Carries the N-Glycan Chain on the Rh Glycoprotein—The susceptibility of the Rh glycoprotein to the endoglycosidases PNGase F and endo-$\beta$-galactosidase has been described elsewhere (Moore and Green, 1987; Mallinson et al., 1990; Avent et al., 1988a; Ridgwell et al., 1994). The Rh50A cDNA sequence contains three consensus N-glycan addition sites, but the topological model for the protein derived from hydroxylation analysis (Ridgwell et al., 1992) suggests that only two of these are extracellular, and therefore likely to carry N-glycan chains (Asn-37 and Asn-355). Expression of the Rh50A cDNA clone in the rabbit reticulocyte lysate cell-free translation system has suggested that only one of these sites may be glycosylated (Ridgwell et al., 1994).

The molecular mass of the largest C-terminal fragment obtained by trypsin treatment of the Rh glycoprotein in erythrocyte membranes (RhGP.T1, 21.5 kDa) did not change when the membranes were derived from erythrocytes pretreated with PNGase F, which suggests that it does not carry an N-glycan chain. However, the PNGase F deglycosylated Rh glycoprotein showed a change in molecular mass from 30.5 to 28.5 kDa after S. aureus V8 protease cleavage in intact erythrocytes, and this

![Proteolysis of Rh glycoprotein in erythrocyte membranes](image-url)

**Fig. 2.** Proteolysis of Rh glycoprotein in erythrocyte membranes. Immunoprecipitation and immunoblotting of Rh-related polypeptides from protease-treated erythrocytes and erythrocyte membranes. a-d, RhGP anti-C immunoblots of material immunoprecipitated by the RhD specific monoclonal antibody AB5 from (a) PNGase F-deglycosylated, S. aureus V8 protease-treated intact erythrocytes; (b) PNGase F-deglycosylated intact erythrocytes; (c) S. aureus V8 protease treated intact erythrocytes; (d) untreated intact erythrocytes. e-k, RhGP anti-C immunoblots of membranes from (e, h, and l) undigested control erythrocytes; (f) trypsin (100 $\mu$g/ml) treated intact erythrocytes; (g and i) trypsin (100 $\mu$g/ml)-treated erythrocyte membranes; (j) PNGase F-deglycosylated erythrocytes; (k) trypsin-treated erythrocyte membranes from PNGase F-deglycosylated erythrocytes. m-o, trypsin (10 $\mu$g/ml) treated leaky erythrocyte membranes from cells of Rh phenotype (m) cDDee; (n) cedde; (o) CCDDee. Molecular mass is in kDa.
Am-37 actually carries an N-glycan chain. The analysis of the Rh50A polypeptide (Ridgwell et al., 1990). The predicted glycosylation sites (Asn-37, Asn-355, and Asn-274) are shown, but only Asn-37 actually carries an N-glycan chain. The S. aureus V8 protease (Glu-34) and trypsin (Lys-196, Arg-323, and Lys-384) cleavage sites are also shown. The C and N termini of the Rh50A polypeptides and the C terminus of the Rh glycoprotein are located in the cytoplasm, and hydrophathy analysis and amino acid sequence alignment of the Rh50A polypeptides and Rh50A glycoprotein suggest that they have the same topology in the membrane. This topological model is therefore likely to be applicable to the family of Rh polypeptides.

The presence of the glycan chain on Asn-37 of the Rh glycoprotein was confirmed by N-terminal sequencing of the 28.5-kDa RhGP.V8 fragment. The N-terminal amino acid sequence obtained from the deglycosylated RhGP.V8 peptide sequence corresponded to amino acids 35–49 of the protein sequence of the Rh50A cDNA, except that Asp was obtained at cycle 3 instead of Asn. This residue corresponds to Asn-37 of the sequence of the intact protein, which is a potential N-glycan addition site. This demonstrates that Asn-37 is normally glycosylated, since PNGase F removes N-glycans from glycoproteins by cleavage of the asparaginyl-glucosamine bond with consequent hydrolysis of the Asn to an Asp (Alexander and Elder, 1982). Glycosylation of Asn-37 may also explain the misassignment of residues 37 and 38 in the original N-terminal amino acid sequence determination of the Rh glycoprotein (Ridgwell et al., 1992).

These results show that the 28.5-kDa S. aureus V8 protease fragment (RhGP.V8) of the Rh glycoprotein carries an N-glycan chain, but that the C-terminal 21.5-kDa trypptic peptide (RhGP.T1) does not. Thus only the most N-terminal of the consensus N-glycan addition site (at Asn-37) is actually glycosylated (Fig. 4). Gavel and von Heijne (1990) examined the frequency of glycosylation of nonglycosylated potential glycan acceptor sites in a large number of glycoproteins. They found that the closer a predicted N-glycan addition site is to the C terminus, the less likely it is to be N-glycosylated. They also noted that the incidence of glycosylation was lower for Asn-X-Ser/N-glycan acceptor sites than for Asn-X-Thr sites. The consensus Rh50A N-glycan acceptor sites at Asn-274 and Asn-355 (Fig. 4) are closer to the C terminus than to the N terminus, and Asn-355 is part of a Asn-X-Thr consensus sequence.

**Location of an Extracellular Loop of the Rh Glycoprotein**—The RhGP.V8 peptide was produced by digestion of intact erythrocytes with S. aureus V8 protease. The protease cleavage site, between residues Glu-34 and Gln-35 of the Rh glycoprotein, must therefore be extracellular. This is consistent with the predicted topology of Rh50A (Fig. 4), which suggests residues 25–56 are located in the most N-terminal extracellular loop.

**Identification of Two Cytosolic Loops of the Rh Glycoprotein**—The Rh glycoprotein does not appear to be susceptible to trypsin cleavage in intact erythrocytes, but trypsin treatment of erythrocyte membranes generated at least 9 trypptic peptides containing the C terminus. The sites of trypsin cleavage which generate these fragments are located in the cytosol. The cleavage sites giving rise to two of these peptides, RhGP.T1 (21.5 kDa) and RhGP.T4 (7–7.5 kDa) were at Lys-196 and Arg-323 of the Rh glycoprotein, respectively.

A 3-kDa C-terminal Rh glycoprotein trypptic peptide was also observed in the total reaction mixture obtained after trypsin treatment of erythrocyte membranes (Fig. 2g). The 3-kDa fragment was not obtained in RhGP anti-C immunoprecipitates derived from trypsin-treated erythrocyte membranes that were washed prior to immunoprecipitation (Fig. 3d), and is therefore not membrane bound. This 3-kDa peptide arises from trypsin cleavage at Lys-384, since there are no other potential trypsin cleavage sites in the Rh50A cDNA sequence that would generate a C-terminal fragment of this size. Lys-384 is therefore likely to be located on the intracellular side of the membrane.

This is further evidence for the cytosolic location of the C terminus of the Rh polypeptides, as amino acid residues 384–409 are hydrophilic (Engelman et al., 1986) and this fragment is clearly not membrane bound.

**Topology of the Rh Family of Polypeptides**—The protein sequences predicted by the Rh30A polypeptide and Rh50A glycoprotein cDNA clones are very hydrophobic and show extensive homology, particularly in the membrane spanning regions. Hydropathy analysis predicts that the proteins have a very similar topology in the membrane. On this basis, experimental evidence for elements of topology in either of the polypeptides is likely to be applicable to both. Our studies suggest that a model for the topology of the Rh family of proteins containing an even number up to 12 membrane spanning domains, as proposed by Avent et al. (1990), is correct. Fig. 4 illustrates this model and shows the location of amino acid residues established in the present study.

**Interaction between the Rh30 Polypeptides and Rh Glycoproteins**—Moore and Green (1987) suggested that the Rh30 polypeptides and Rh glycoproteins interact to form a complex in the erythrocyte membrane. The different Rh antigens expressed on red cells depend on sequence differences in the Rh30 polypeptides and not the Rh glycoproteins (Ridgwell et al., 1992). The changes in the trypsin digestion patterns of the Rh glycoprotein in cells of different Rh phenotype (Fig. 2, l–o) suggest that the structure of the Rh glycoprotein may be slightly different when it is associated with different Rh30 polypeptides. This is consistent with the observation that the Rh glycoprotein associated with different members of the Rh30 polypeptide family shows different surface labeling patterns (Moore and Green, 1987; Avent et al., 1988a, 1988b; Barker et al., 1992), although the Rh glycoprotein molecule involved appears to be the same in each case (Ridgwell et al., 1994).

Our proteolysis studies have located one region of interaction between the Rh30 polypeptide and Rh glycoprotein in the Rh complex. The 28.5-kDa RhGP.V8 peptide was co-precipitated with the Rh30 polypeptides from S. aureus V8 protease-treated erythrocytes using Rh-specific monoclonal antibodies (Fig. 2a). However, trypptic peptides containing the Rh glycoprotein C terminus, including RhGP.T1 (which results from trypsin cleavage at Lys-196 of the Rh glycoprotein), were only immunoprecipitated by the RhGP anti-C polyclonal antibody (Fig. 3, a–d) and not by the Rh-specific monoclonal antibodies. The absence of Rh glycoprotein trypptic fragments from the monoclonal antibody immunoprecipitates was not due to the destruction of the antibody epitopes by trypsin treatment of the membranes.
as the antibodies still immunoprecipitated the Rh30 tryptic polypeptides under these conditions (Fig. 3, h, i, k, and l). These results suggest that the Rh30 polypeptide interacts with the Rh glycoprotein between the extracellular S. aureus V8 protease cleavage site (Glu-34) and the cytoplasmic trypsin cleavage site (Lys-196) of the Rh glycoprotein. This region encompasses five predicted transmembrane helices (helices 2-6) in the N-terminal half of the Rh glycoprotein. We do not know whether the region between the N terminus and Glu-34 of the Rh glycoprotein, which contains the first transmembrane helix, is also immunoprecipitated by the Rh-specific monoclonal antibodies from S. aureus V8 protease-treated red cells.

Although it is known that genetic differences in the Rh30 polypeptides rather than the Rh glycoprotein specify the different Rh blood group antigens (Ridgwell et al., 1992), the sites on the Rh proteins reactive with Rh-specific antibodies, such as anti-D or the R6A type of monoclonal antibodies have not been defined. It is likely that they interact only with the Rh30 polypeptides, in which case the Rh glycoprotein would be co-immunoprecipitated with the Rh30 polypeptide because of a direct association between the N-terminal half of the Rh glycoprotein with the Rh30 polypeptide. However, it is also possible that some part of the Rh glycoprotein is involved together with the Rh30 polypeptide in the binding site of the Rh-specific antibodies. The co-immunoprecipitation of the Rh30 polypeptides and the Rh glycoprotein could result from the formation of an antibody bridge between the Rh30 polypeptides and the N-terminal half of the Rh glycoprotein. In either case our results lead us to conclude that the N-terminal portion of the Rh glycoprotein is located physically close to the Rh30 polypeptides.

A Model for the Organization of the Rh Complex—There is considerable evidence that the Rh30 proteins and Rh50 glycoproteins are present as a complex in the erythrocyte membrane (Moore and Green, 1987; Avent et al., 1988a, 1988b). The Rh30 polypeptides and the Rh glycoprotein appear to form the core of the complex, with the additional involvement of glycophorin B, and possibly the CD47 glycoprotein, blood group LW antigens, and Fy glycoprotein (reviewed by Agre and Cartron (1991) and Anstee and Tanner (1993)). Hartel-Schenk and Agre (1992) determined the size of the complexes containing the Rh-association proteins in nonionic detergent-solubilized membranes, and estimated that they contain 170 kDa of protein in correction for bound detergent. This size is consistent with the complex being based on an αβ-type tetramer made up of two Rh30 polypeptide molecules and two Rh glycoprotein molecules. The additional proteins such as glycophorin B which are probably also present will not be considered in the following discussion.

A model for the organization of the Rh30 polypeptides and Rh glycoprotein in the Rh complex is shown in Fig. 5. The N- and C-terminal halves of the Rh glycoprotein are shown as comprising two domains within the lipid bilayer, separated by the trypsin cleavage site at Lys-196, each containing six transmembrane segments. Recent evidence for the presence of subdomain structures located in the lipid bilayer in multispanning membrane proteins has come from structural studies on two-dimensional crystals of the sarcoplasmic reticulum calcium pump (Toyoshima et al., 1993), and of human red cell band 3 (Wang et al., 1993).

We assume that the sequence and topological homology between the Rh30 polypeptide and Rh glycoprotein extends to their tertiary structure and that both proteins have similar domain structures. Consistent with this, the Rh30 polypeptide (in this case the molecule(s) carrying CcEe antigens reactive with the R6A-type antibodies) is cleaved by trypsin into two similar sized fragments (Fig. 3, g-i), as has been previously observed by Suyama and Goldstein, (1992). This cleavage is probably in the cytosolic loop at Lys-189, Lys-193, or Arg-201 of the Rh50A protein, and also divides this protein into two halves each containing six membrane spanning segments. We suggest that the N-terminal half of the Rh glycoprotein interacts with the homologous N-terminal half of the Rh30 polypeptide, so that these portions of the proteins form the center of the complex. It is interesting that both the N- and C-terminal halves of the Rh30 polypeptide are immunoprecipitated from trypsin-treated membranes by BRIC 69 and BRIC 207, the monoclonal antibodies of the RhA type (Fig. 3, g-i). It may be that the N- and C-terminal halves of the Rh30 polypeptide associate together more strongly than the corresponding portions of the Rh glycoprotein. However, another possibility is that the binding sites of the R6A type of monoclonal antibodies comprise surface loops on the Rh30 polypeptide derived from both the N- and C-terminal halves of the molecule, and the monoclonal antibody acts as a bridge which stabilizes the association between the two halves of the Rh30 polypeptide. Resolution of this question and validation of the model in Fig. 5 will clearly require further investigation.

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