Immunoochemical Analysis of the Human Erythrocyte Rh Polypeptides*

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We have used rabbit polyclonal antisera raised against synthetic peptides complementary to different domains of the Rh polypeptides and Rh glycoprotein to examine the topography and organization of these proteins in the human erythrocyte membrane. Previously unrecognized exofacial protease sites have been identified on Rh CcEe, D proteins, and Rh glycoprotein. The Rh D protein has two specific bromelain cleavage sites located within the first and sixth predicted external domains, with the site of cleavage localized in the sixth domain to lie between residues 353 and 354. All Rh polypeptide species were found to be susceptible to cleavage with trypsin and subtilisin within the first external domain of these proteins. The Rh glycoprotein has two bromelain cleavage sites within the first external domain. These flank the single N-glycosylation site (Asn37), with the cleavage site toward the C-terminal end of the domain. These data indicate that Rh glycoprotein is susceptible to deglycosylation by the enzyme bromelain.

Immunoprecipitation experiments have revealed that anti-C, -c,E, -e, and -D immune complexes are reactive with antigen CcEe, confirming expression data (20). We also define trypsin cleavage sites on both the Rh proteins to lie between residues 353 and 354. All Rh polypeptide species were found to be susceptible to cleavage with trypsin and subtilisin within the first external domain of these proteins. The Rh glycoprotein has two bromelain cleavage sites within the first external domain. These flank the single N-glycosylation site (Asn37), with the cleavage site toward the C-terminal end of the domain. These data indicate that Rh glycoprotein is susceptible to deglycosylation by the enzyme bromelain.

**Materials and Methods**

Human Erythrocytes and Antibodies—Erythrocytes of the common Rh phenotypes (i.e. CDe, cDe, and cDe; the Fisher-Race Rh CDe nomenclature being used throughout this report) were obtained from the National Blood Service, South West. Erythrocytes of Rhnull phenotype (donor K. W.) were obtained from Dr. Sokol, National Blood Service, Sheffield, U. K. and of -D- phenotype from Dr. M. Fischer, Regional Transfusion Centre, Oxford, U. K. (donor G. L.); and R. Wójcicki, American Red Cross, St. Paul, MN (donor ShMa). Monodonal anti-D (AB-5) was from Dr. Belinda Kumpel, International Blood Group Reference Laboratory, and monoclonal anti-c (MS-242 and MS-257), anti-c (MS-45), and anti-e (MS-235) were from Dr. Keith Thompson, Institute of Immunology and Rheumatology, Oslo, Norway. Monodonal antisera were also obtained from Dr. Douglas Voak, National Blood Service, Cambridge.

Synthetic Peptides and Rabbit Polyclonal Antibodies—Synthetic pep-
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Anti-Nt

Washed erythrocytes of different Rh phenotypes were digested with papain as described under "Materials and Methods" and membranes produced by hypotonic lysis. Rh phenotypes are indicated above pairs of tracks, and undigested (−) and digested (+) membrane preparations are similarly shown.

Anti-Ct

Peptides were coupled to keyhole limpet hemocyanin and bovine serum albumin using cysteine residues attached to the N termini and C termini of the Rh glycoprotein were as described (13, 14, 23). Peptides were coupled to keyhole limpet hemocyanin and bovine serum albumin using cysteine residues attached to the N termini and C termini of the peptides and antisera generated as described (13).

Immunoblotting—Immunoblotting was as described by Mallinson et al. (24), except that SDS-PAGE\(^1\) was performed using either the Tricine buffer system of Schagger and von Jagow (25) or the discontinuous system of Laemmli (26). Antisera were diluted 1:50 to 1:100 in Dulbecco's modified Eagle's medium with 10% fetal calf serum prior to immunoblotting or in PBS, 0.2% (v/v) Tween 20, 5% (w/v) bovine milk powder. Detection of bound antibody was achieved by addition of a 1:250 dilution of a swine anti-rabbit horseradish peroxidase conjugate (Dako, Copenhagen, Denmark; P217), diluted in PBS, 0.2% (v/v) Tween 20, 5% (w/v) bovine milk powder.

Bromelain and Papain Digestion of Erythrocytes—Washed erythrocytes were incubated at 37°C with 3 mg/ml bromelain (Sigma, Poole, Dorset, U.K.) in PBS, pH 7.4, for 1 h. Cells were then washed three times in 25 volumes of PBS, pH 7.4, once in 0.1 M NaH\(_2\)PO\(_4\) before either immunoprecipitation by the method of Moore et al. (4) or the production of membranes by hypotonic lysis and repeated washing in ice-cold 5 mM NaH\(_2\)PO\(_4\), pH 8.0. Papain digestion was performed on washed erythrocytes by incubating cells with 50 μl of an activated papain preparation (BPL Diagnostics, Elstree, Herts., U.K.).

Subtilisin and Trypsin Digestion of Erythrocytes—Subtilisin (Subtilisin Carlsberg, Sigma, Poole, Dorset, U.K.) was used at a concentration of 3 mg/ml washed erythrocytes in PBS, pH 7.4. Tryptsin digests (TPCK-treated, Sigma) were performed at a concentration of 1–3 mg/ml erythrocytes in two different digest buffers, PBS, pH 7.4, for 1 h at 37°C, or at low ionic strength conditions in a 5% (v/v) suspension of erythrocytes in 300 mM sucrose, 5 mM NaH\(_2\)PO\(_4\), pH 8.0. Digests were performed for 1 h at 37°C and were terminated by four washes of the erythrocytes in 25 volumes of PBS to remove excess protease. Erythrocyte membrane ghosts were then produced by hypotonic lysis.

Isolation and N-terminal Amino Acid Sequence Analysis of 30-kDa Rh D and Rh Glycoprotein Bromelain Peptides—200 ml of washed, packed phenotype cDe erythrocytes were digested with 1 mg/ml bromelain for 2 h at 37°C, washed three times with 5 volumes of PBS, pH 7.4, and were then immunoprecipitated with 5 volumes of monoclonal anti-D (AB-5). Immunoprecipitated samples were separated on a 12% (w/v) SDS-PAGE Tricine gel (25). The gel components were then transferred onto Problot membrane (Applied Biosystems International, Warrington, U.K.), bands were visualized by staining the membrane with Serva blue G (0.1% w/v) in 50% (v/v) methanol, and destained with 50% (v/v) methanol. Peptides were identified by immunoblotting a small fraction of the immune complexes with anti-Nt, anti-Ct, and anti-Rh30A loop 1 and anti-Rh glycoprotein Cter antisera. Stained bands were excised and loaded onto the blot cartridge of an Applied Biosystems 477A protein sequencer and sequenced.

RESULTS

Immunoblotting of Erythrocyte Membranes Derived From Individuals Expressing Different Rh Phenotypes Using Rabbit Anti-loop 1 and 4 Antibody—All rabbit antisera reacted with peptide coupled to bovine serum albumin as determined by dot-blot analysis (not shown). All rabbit antisera reacted with erythrocyte membrane components of 35 kDa on immunoblot analysis in all membranes from individuals of common Rh phenotypes (Cde, cDe, cde, Cde, Cde). Reactivity of anti-loop 4e and 4e sera was identical with all membranes tested regardless of Rh phenotype. All sera failed to react with erythrocyte membrane derived from a Rhnull donor (K. W.) (not shown). Preimmune bleeds obtained from both rabbits failed to react with any erythrocyte membrane component as determined by immunoblot analysis (not shown).

Identification of Rh D-specific Bromelain/Papain Sites—Immunoblot analysis of erythrocyte membranes derived from bromelain or papain-treated intact erythrocytes using anti-Nt and Anti-Ct sera revealed the presence of peptides of 6 kDa in Rh D-positive membranes only (papain digests illustrated in Fig. 1; bromelain digests not shown). Weakly immunostaining bands of ~6 kDa were detected in all erythrocyte membrane preparations when using anti-Ct (Fig. 1), which may indicate autolysis of Rh polypeptides during the preparation procedure. When blotting was performed using anti-Nt on both Rh D-positive membranes and anti-D immunoprecipitates derived from bromelain-treated intact Rh D-positive erythrocytes, two Rh D-specific immunostaining bands were detected at 31 and 6 kDa (membranes shown in Fig. 1, immunoprecipitates shown in Fig. 2). A papain-resistant band of molecular mass 35 kDa was present in all membranes regardless of Rh phenotype (Fig. 1). Immunoblot analysis of anti-D immunoprecipitates revealed that only one (6 kDa) immunostaining band was detected when using anti-Ct (Fig. 2). Two Rh D protein-derived peptide bands were identified when immunoblotting was performed using anti-Nt on the same immunoprecipitates (Fig. 2).

\(^1\) The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TPCK, tosylphenylalanyl chloromethyl ketone; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
and also when membranes derived from bromelain-treated erythrocytes of two individuals (B. B. and ShMa) with the -D- phenotype (which lack the Rh CcEe antigens) were used (Fig. 3). A third individual with the -D- phenotype (G. L.) gave a different immunoblot pattern with bands of 11.5 and 6 kDa detected with the anti-Nt antiserum (Fig. 3). Time course digestion experiments, utilizing anti-D immunoprecipitation from bromelain-treated erythrocytes of phenotype cDE, and immunoblot analysis of these immunocomplexes with anti-Nt, revealed that the immunostaining of two peptides of 31 and 6 kDa was constant throughout an 8-h digestion period. However, digestion at time periods longer than 8 h resulted in significant red cell lysis, with resultant failure of the immunoprecipitation with anti-D (not shown).

Immunoblot analysis of membranes derived from bromelain-treated erythrocytes following SDS-PAGE using Tricine-buffered gels with anti-loop 1 serum showed weak immunostaining of the 6-kDa band (not shown). In contrast, two bands of 28 and 25 kDa were detected in anti-D immunoprecipitates derived from bromelain-treated erythrocytes when immunoblotted using anti-loop 1 (Fig. 2). These bands are consistent with existence of Rh D proteins in these immunoprecipitates devoid of both the N- and C-terminal domains and C-terminal domain only. The Rh proteins were found to migrate notably slower in Tricine gels than in Tricine gels.

Amino Acid Sequence Analysis of Rh D Polypeptide Bromelain Peptides—Preparative immunoprecipitation using anti-D and bromelain-treated erythrocytes of phenotype cDE was followed by transfer to Problott sequencing membrane. The identification of the N-terminal sequences of three of the peptides contained in these immunoprecipitates was possible following Serva blue G staining of the blot (Fig. 4). One Rh D peptide species contained in the anti-D immunoprecipitate (peptide 2, Fig. 3) has revealed that the N-terminal amino acid of this 6.2-kDa peptide (containing the C-terminal domain of the Rh D polypeptide) is equivalent to residue 354 of the Rh D polypeptide sequence (10, 11) (see Fig. 8). Precise definition of the presumed bromelain cleavage sites toward the N terminus of the Rh D protein (i.e. within the first external domain) could not be determined, as these peptides comigrated with a 30–35-kDa Rh glycoprotein peptide.

Amino Acid Sequence Analysis of Rh Glycoprotein Bromelain Peptides—The N-terminal amino acid of the 30–35-kDa Rh glycoprotein bromelain peptide (peptide 1 in Fig. 4) corresponded to residue 40 of the Rh glycoprotein sequence (17). This peptide was shown to contain the C terminus of the Rh glycoprotein by immunoblot analysis of this preparative immunoprecipitate with anti-Rh glycoprotein Cter serum (not shown). A weak Coomassie staining peptide of 6 kDa corresponding to an Rh glycoprotein peptide (sequence NH2 MRFT-FPLMAIVLEIAM) was identified in the preparative immunoprecipitates (peptide 3, Fig. 4), which matches the predicted N-terminus sequence of the published Rh glycoprotein sequence (17).

Subtilisin Treatment of Erythrocytes of Different Rh Phenotypes—Washed erythrocytes were digested with 3 mg/ml subtilisin, separated by SDS-PAGE using the Tricine buffer system (25) on 12% (w/v) polyacrylamide gels, and immunoblotted with rabbit antisera (anti-loop 4e; anti-Ct and anti loop 1). When anti-loop 4e was used, bands of 30 kDa were detected in membranes derived from subtilisin-digested erythrocytes (Fig. 5). In membranes derived from no enzyme control digests, intact 32–35-kDa Rh proteins were found as expected. Immunostaining bands of 6–7 kDa were detected in immunoblots of subtilisin-treated membranes when anti-loop 1 serum was used (Fig. 5). The molecular size of this peptide indicates the
existence of a subtilisin cleavage site within the first extracellular domain of all Rh proteins. Immunoblot analysis using anti-Nt revealed that a band of the same mobility was also detected using this antiserum (not shown). A band of 5.0 kDa was detected in D-positive membranes only when anti-Ct was used (Fig. 5). This is consistent with the existence of a subtilisin cleavage site in the sixth extracellular domain of the Rh D protein. Time course digestion experiments showed that subtilisin digestion was essentially complete within 3 h with all Rh proteins being subtilisin-sensitive in all Rh phenotypes tested (not shown).

Trypsin Treatment of Erythrocytes of Different Rh Pheno-
types—Digestion of erythrocytes with TPCk-treated trypsin at normal and low ionic strength conditions followed by immunoblot analysis (with anti-loop 1 serum) of the derived membranes revealed that an immunostaining band of 6–7 kDa was detected (Fig. 6). This is consistent with the existence of a trypsin site in the first extracellular domain of the Rh polypeptides. Immunostaining bands of 30 and 35 kDa were detected in immunoblots when anti-Ct and anti-loop 4e were used (Fig. 6). This is consistent with only a single trypsin site being present on the Rh polypeptides. A band of 35 kDa was detected in all membranes derived from trypsin-treated cells regardless of Rh phenotype, indicating the presence of a trypsin-resistant Rh polypeptide in all cell membranes tested. Time course digestion experiments revealed that the trypsin-resistant band remained up to a 5-h digestion in both normal and low ionic strength digest buffers (not shown).

Immunoblot Analysis of Anti-C, -c, -D, -E, and -e Immune Complexes Using Anti-loop 4e and Anti-Ct Rabbit Antisera—Immunoprecipitation was performed using monoclonal anti-C, -c, -D, -E, and -e antibodies on erythrocytes of known Rh phenotypes as described under “Materials and Methods.” Immune complexes were then separated by SDS-PAGE using the Laemmli buffer system (26) on 10% (w/v) polyacrylamide gels and immunoblotted with rabbit antisera. All immune complexes examined were found to contain Rh proteins which possess the C-terminal domain and the fourth extracellular domain of Rh proteins (Fig. 7). These results are consistent with Rh C, c, D, E, and e antigens being expressed on full-length polypeptide chains. Weakly immunostaining bands were observed with anti-E and anti-e immune complexes, indicating inefficient immunoprecipitation with these antibodies (not shown).

**DISCUSSION**

These studies provide direct evidence for the organization and topology of the Rh proteins at the erythrocyte surface. We provide evidence to unequivocally identify exofacial regions of the Rh proteins based on amino acid sequence analysis of immunopurified peptides derived from protease-treated erythrocytes and demonstrate that Rh C/c antigen expression does not occur on shortened Rh polypeptide isoforms. The rabbit sera described here react strongly with membranes derived from erythrocytes expressing normal Rh phenotypes. No difference in reactivity was found when immunoblotting was performed with anti-loop 4e or 4E sera. Hermand et al. (27) report a rabbit sera MPC-4 that was raised to a synthetic peptide complementary to the fourth loop of Rh polypeptides. This sera reacts strongly with Rh E-positive membranes but only weakly with Rh e-positive membranes. We assume the difference in reactivity of our sera and MPC-4 is due to the size of peptide immunogen.

Two Rh D protein-specific protease sites have been localized. A bromelain/papain cleavage site (that was originally identified toward the C-terminal end of the molecule (13, 28)) is located between residues 353 and 354 (Figs. 4 and 8). A second bromelain site is located within the first external domain, although we were unable to precisely define its position. This site is unexpected inasmuch as the Rh D (Rh30D) polypeptide amino acid sequence (inferred from its cDNA sequence) in this domain is identical to the Rh CcEe (Rh30A) protein. However, protein sequence analysis of immunopurified Rh D proteins have shown several differences in this region of the protein when compared with the Rh CcEe proteins. The identification of an Rh mRNA species, Rh30B, confirmed the authenticity of these changes (8). It is possible that these amino acid changes create this bromelain site and suggest the possibility that the Rh D protein complex is heterotetrameric, composed of single Rh30D and Rh30B subunits and two Rh glycoprotein subunits. Evidence for the existence of a tetrameric Rh "core complex" has been obtained by velocity sedimentation studies (29). Alternatively the Rh D-specific cleavage may be generated by different degrees of association of Rh D proteins with the Rh glycoprotein as compared with Rh CcEe proteins. It is likely that Rh protein-Rh glycoprotein interactions occur via N-terminal domain association (14), which indicate the involvement of this domain of the Rh proteins. Further characterization of the Rh30B protein and Rh D protein complex as a whole should
Both subtilisin and trypsin cleavage result in the loss of a single membrane spanning domain from the Rh polypeptides. Bromelain treatment results in the loss of one or two membrane spanning domains. Molecular weight shifts of predicted size are observed in enzyme-digested samples following immunoblotting (Figs. 1–3 and 5–6). This indicates that the resolution of the SDS-PAGE utilized in these studies is sufficient to resolve Rh protein fragments that differ in size by 50 amino acid residues or less (41 in the case of trypsin digests). Shortened Rh protein isoforms (proposed to express Rh C/c antigens (19, 22)) would be greater than 100 amino acids smaller than full-length Rh proteins and should easily be resolved by SDS-PAGE. Furthermore, we provide unambiguous evidence that immunopurified Rh C and c proteins are reactive with rabbit sera raised against the fourth external loop and C-terminal domains of Rh proteins (Fig. 7). These domains would not be present on the proposed isoform structures. Our results suggest that the transcripts encoding the shortened polypeptides are a result of missplicing, and the polypeptide products are not inserted into the membrane in a stable form.

Our data support the hypothesis that the Rh C/c and E/e antigens are carried on the same polypeptide chain. Recent in vitro expression studies also support this hypothesis. Expression of an Rh E/e transcript (Rh30A) in K562 cells following retroviral transduction results in elevated expression of Rh C and E antigens. This indicates that a single full-length Rh transcript (Rh30A) can encode both Rh c and E antigens simultaneously (20). A strong argument against the expression of Rh Cc and Ee antigens on the same polypeptide backbone is that the abundance of C/c antigens sites is almost 2-fold greater than E/e sites on the same erythrocyte (30, 31). It can be argued that steric constraints may depress the apparent concentration of E/e antigen sites. Our observations indicating the N-terminal half of the Rh CcEe proteins (where residues critical for C/c expression are known to reside (19); namely Ser/Pro103) is susceptible to proteolysis reflect that external domains of Rh proteins have different accessibility.

Two bromelain sites have been identified on the first external domain of the Rh glycoprotein. The first occurs between residues 39 and 40 (Figs. 4 and 8), and it would appear that the second occurs toward the N-terminal side of the N-glycosylation site at Asn37. This assumption can be made because the identification of a small peptide corresponding to the N-terminal sequence of the Rh glycoprotein has been found in preparative anti-D immunoprecipitates from bromelain-treated cells (peptide 3, Fig. 4). This peptide is of insufficient size to carry the Rh glycoprotein N-glycan chain which is at least 25 kDa in size (14). Clearly bromelain treatment deglycosylates the Rh glycoprotein, and the removal of the bulky N-glycan moiety may explain the increased sensitivity of enzyme-treated cells in red cell serology when using anti-D.

These studies clearly demonstrate that the Rh proteins and glycoprotein form a tightly packaged multi-subunit complex in the erythrocyte membrane. The co-precipitation of proteolytic fragments of both Rh D protein and Rh glycoprotein (Fig. 4) indicates that its integrity is still grossly intact following external proteolysis. This indicates that it is likely that there is considerable interaction of the membrane domains of both Rh proteins and glycoproteins in this complex. Major alterations to individual Rh complex components are likely to be extremely detrimental to its correct assembly during biosynthesis. Large deletions that have been described from Rh transcripts which result in altered reading frame and/or truncated Rh polypep-
tide structures are most likely degraded following their synthesis. The experiments described here provide considerable evidence that these aberrant protein structures are not expressed at the erythrocyte surface.

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