

Immunochemical Characterization of Rhesus Proteins With Antibodies Raised Against Synthetic Peptides

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Rabbit polyclonal antibodies were raised against synthetic peptides corresponding to hydrophilic regions of the human Rhesus (Rh) IX cDNA-encoded polypeptide predicted to be extracellularly or intracellularly exposed in the topologic model of the Rh blood group protein. Four antibodies encompassing residues 33-45 (MPC1), 224-233 (MPC4), 390-404 (MPC6), and 408-416 (MPC8) were characterized and compared with a polyclonal anti-Rh protein obtained by immunization with purified Rh proteins. All antibodies had specificity for authentic Rh polypeptides and reacted on Western blot with Rh proteins immunoprecipitated with human monoclonal anti-RhD, -c, and -E. MPC1, but not the other antibodies, agglutinated all human erythrocytes except Rh_{null} and Rh_{mod} cells, which either lack totally or are severely deficient in Rh proteins, respectively. Immunoblotting analysis with membrane proteins from common and rare variants showed that MPC1 and MPC8 reacted in Western blot with 32-Kd Rh polypeptides from all common red blood cells except those from Rh_{null} and Rh_{mod}, indicating that peptide regions 33-45 and 408-

416 may be common to several if not all Rh proteins, whatever the Rh blood group specificity. MPC4 reacted only with membrane preparations from cells carrying the E antigen, whereas MPC6 recognized preferentially the Rh proteins from E and Ee preparations, suggesting that the protein encoded by the RhIXb cDNA carries the E and/or e antigen(s). Immunoabsorption experiments using inside-out or right-side-out sealed vesicles from DccEE red blood cells as competing antigen showed that the MPC6 and MPC8 antibodies bound only to the cytoplasmic side of the erythrocyte membrane, thus providing evidence for the intracellular orientation of the C-terminal 27 residues of the Rh polypeptides. Attempts to transiently or stably express the Rh cDNA in eukaryotic cells were largely unsuccessful, suggesting that Rh antigen expression at the cell surface requires correct transport and/or folding of the Rh proteins, possibly as a complex with one-membrane proteins of the Rh cluster that are lacking in Rh_{null} cells.

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THE RHESUS (Rh) blood groups were discovered more than 50 years ago^{1,2} and were found to play an important role in transfusion medicine.³ However, our current knowledge on the biochemistry of Rh proteins and their genes has developed only recently.⁴ Indeed, at least three distinct integral membrane proteins of apparent molecular weight (Mr) 32,000 that are nonglycosylated⁵ but fatty acylated⁶ carry the polymorphic Rh antigens. These proteins are highly homologous⁷ and relatively well conserved between species.⁸ It is known, in addition, that free sulfhydryl groups are required for RhC and D antigenic expression.^{9,10} After the cloning of a cDNA encoding one of the Rh proteins,^{11,12} further studies by Southern blot analysis¹³ indicated that the RH locus, which is located on chromosome 1p32-p36,^{14,15} is composed of two related genes (*D* and *CcEe*) in "Rh-positive" individuals (defined as carriers of the major RhD antigen), but of a single gene (*CcEe*) in "Rh-negative" individuals (defined as nonproducers of D antigen). The *CcEe* gene transcript that was first cloned^{11,12} produces both the Cc and Ee proteins, possibly by alternative splicing,¹⁶ whereas the *D* gene transcript was cloned only very recently.¹⁷ Sequence comparison showed that both genes are highly homologous and are most likely derived by duplications of an ancestral gene during evolution.

Despite these advances, the antigenic specificity of the Rh protein encoded by the first Rh gene cloned^{11,12} has not been established. This report describes the preliminary immunochemical studies of the Rh protein encoded by this clone using polyclonal antibodies against synthetic peptides deduced from the predicted coding sequence. These antibodies were also used to probe the topologic organization of the Rh proteins within the red blood cell (RBC) membrane.

MATERIALS AND METHODS

RBCs and monoclonal antibodies (MoAbs). Freshly collected RBCs and rare cryopreserved erythrocyte variants typed in the RH blood group system were from the Centre National de Référence

sur les Groupes Sanguins (CNRGS; Paris, France). Human and murine MoAbs used as anti-D, -c, and -E,¹⁸ R6A,¹⁹ BS58,²⁰ and 2D10²¹ have been already described. Two other human MoAbs, anti-e (IgM) and anti-C (IgM), were generous gifts from the CTS of Nantes and Lille, respectively.

Peptides and polyclonal antibodies. Peptides were selected from hydropathy plot analysis and antigenicity index calculations²² based on the primary structure of the protein encoded by the RhIXb cDNA clone.¹¹ The nomenclature and position of these peptides were as follows with respect to the mature protein: pRh2, 1-10; pRh6, 33-45; pRh14, 66-76; pRh5, 102-113; pRh7, 153-164; pRh4, 185-202 (with an Asn → Leu substitution at position 197); pRh8, 224-233; pRh9, 256-266; pRh10, 280-288; pRh11, 307-318; pRh12, 338-345; pRh3, 390-404; and pRh13, 408-416. Variants of the pRh8 were also used: pRh8, (Pro → Ala at position 225), based on the Rh4 isoform transcribed from the *CcEe* gene,¹⁶ and pRh8₂ (substitutions, Pro → Ala at position 225 and Gln → Glu at position 232), based on the RhD protein sequence.¹⁷ The peptide variant pRh4, (substitutions, Lys → Glu at position 192, Asn → Lys at position 197, and Arg → Thr at position 200) was also based on the RhD protein sequence.¹⁷

All peptides were synthesized using the f-moc (N-9-fluorenyl-

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Table 1. Properties of Polyclonal Anti-Rh Antibodies

Rabbit Antibodies	Peptide Detected (ELISA)	Agglutination Titer		Rh Proteins Detected (Western blot) [†]		Epitope Sidedness (vesicle binding)
		Rh Commun (D* or non-D)	Rh Null	Membrane Preparations	Immuno-purified	
MPC1	pRh 6 (33-45)	320	0	All	D*, c; E	Out
MPC4	pRh 8 (224-233) [‡]	0	0	E	E	NT
MPC6	pRh 3 (390-404)	0	0	E/e	E	In
MPC8	pRh 13 (408-416)	0	0	All	NT	In
Anti-Rh protein	pRh 4 (185-202) [§]	0	0	All	NT	NT

Abbreviation: NT, not tested.

* D and D variants: D --, Dc --, D^{IV}, D^V, D^{VII}.

[†] Rh proteins were detected by Western blot either from membrane preparations or from immunoprecipitates.

[‡] Single point mutations at position 225 (Pro → Ala, peptide pRh 8₁) or 232 (Gln → Glu, peptide pRh 8₂) abolish reactivity.

[§] Peptide pRh 4₁ with substitutions at positions 192, 197, and 200 is unreactive.

methoxycarbonyl) protocol²³ by Neosystem (Strasbourg, France) or the CTS Lille (Lille, France). For use as antigens, the peptides were coupled to keyhole limpet haemocyanin (KLH) using the bisdiazobenzidine, glutaraldehyde²⁴ or the N-maléimido benzoic acid N-hydroxysuccinimide ester²⁵ protocols according to the reactive groups present on each peptide. When necessary, a tyrosine or a cysteine was added either at the N-ter or C-ter position to carry on the coupling reactions. The conjugates (0.3 mg/mL in phosphate-buffered saline [PBS]) were emulsified in Freund's complete adjuvant and injected subcutaneously (500 μ L) into male rabbits ("Fauve de Bourgogne"). Seven similar injections over a period of 30 weeks were performed and antisera were started to be collected 3 weeks after the third injection. Antibody titer and peptide specificity were determined by a solid-phase enzyme-linked immunobinding assay (ELISA) using synthetic peptides (50 ng/well) coated to 96-well polyvinylchloride microplates (Falcon type III, Becton Dickinson, Oxnard, CA) in 0.1 mol/L carbonate-bicarbonate buffer, pH 9.6 for 16 hours at 4°C. Washed microplates were saturated with 2% (wt/

vol) bovine serum albumin (BSA) in ELISA buffer (140 mmol/L NaCl; 2.7 mmol/L KCl; 6.5 mmol/L Na₂HPO₄, and 1.4 mmol/L NaH₂PO₄, pH 7.2) for 1 hour at 37°C. A 100- μ L rabbit antiserum aliquot diluted in PBS was added to each well and incubated for 3 hours at 37°C. After three washes in ELISA buffer containing 0.05% (wt/vol) Tween 20, bound antibodies were shown with a goat antirabbit alkaline phosphatase conjugate (BioAtlantique, Nantes, France; diluted 1:2,000) and alkaline phosphatase substrate kit (BioRad, Rockville, NY). The anti-Rh protein antibody obtained by immunizing rabbits with a purified RhD protein preparation has already been described.²⁶

Protein chemistry. RBC membranes from common and rare Rh variants were prepared by hypotonic lysis²⁷ and immunoprecipitations with human or murine MoAbs were performed as described by Bloy et al.²⁸ For Western blot analysis,²⁹ RBC membrane proteins were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose sheets (Schleicher and Schull, Keene, NH; 0.1 μ m). After immunostaining with rabbit polyclonal antibodies, bound antibodies were detected with alkaline phosphatase-labeled goat anti-rabbit IgG (1:800) and the alkaline phosphatase substrate kit (BioRad).

Immunoabsorption assays. Right-side-out vesicles (ROVs) and inside-out vesicles (IOVs) were prepared from human Rh-positive RBCs (DccEE phenotype) and purified through a barrier of 8% (wt/vol) dextran T70.³⁰ For immunoabsorptions, 50 μ L of each polyclonal antibody was mixed with 4 vol of sealed vesicles at the concentration of 2.5 mg of protein/mL. After 2 hours at 37°C with constant mixing, the vesicles were pelleted by centrifugation at 20,000g for 15 minutes and extensively washed. Free antibodies remaining in the supernatants and bound antibodies eluted from the vesicles by the digitonin method³¹ were analyzed by Western blot and solid-phase ELISA.

RESULTS

Polyclonal antibodies raised against synthetic peptides corresponding to hydrophilic regions of the Rh protein encoded by the RhIXb cDNA clone¹¹ were used to investigate the membrane topology of the Rh polypeptide as well as the structural basis of the Rh antigen reactivity. Four peptides (pRh6, residues 33-45; pRh8, residues 224-233; pRh3, residues 390-404; and pRh13, residues 408-416 of the mature protein) that elicited a strong immune response (antibody MPC1, MPC4, MPC6 and MPC8, respectively) were tested

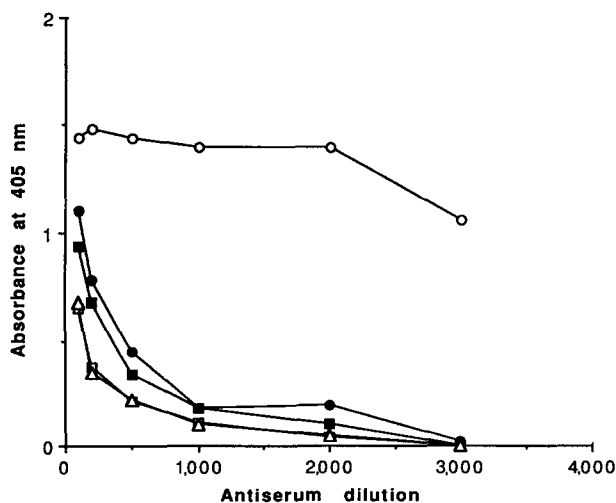


Fig 1. Specificity of MPC4 antibody. The MPC4 antiserum was tested in the ELISA assay (see Materials and Methods) with peptide pRh8 (○) and its variants pRh8₁ (●) (Pro → Ala at position 225) and pRh8₂ (■) (substitution, Pro → Ala at position 225 and Gln → Glu at position 232); pRh6 (□) and pRh10 (△) peptides are used as negative controls. Each point is the mean of triplicate determinations.

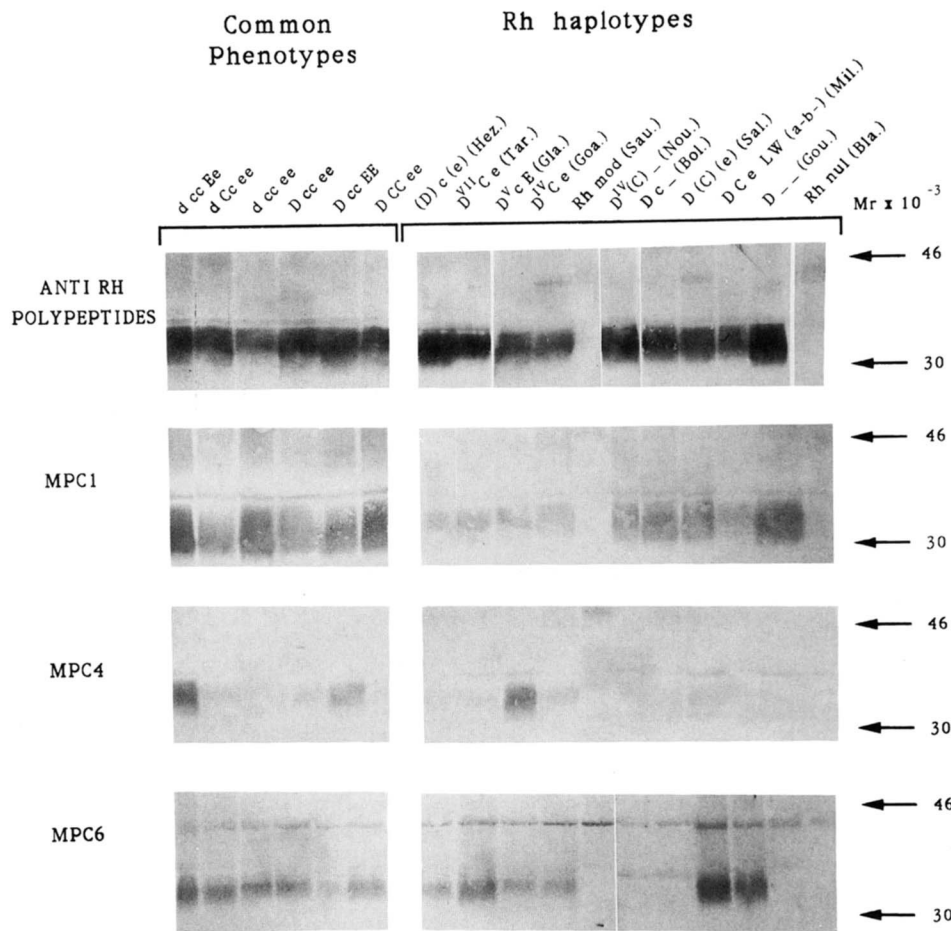


Fig 2. Immunostaining of RBC membrane proteins from common and rare Rh phenotypes. Total membrane proteins (120 μ g) separated on SDS-PAGE (11% polyacrylamide) were transferred onto nitrocellulose sheets and incubated respectively with the anti-Rh polypeptide (1:1,000), MPC1 (1:4,000), MPC4 (1:800), and MPC6 (1:800). Washings were followed by incubation with goat antirabbit IgG conjugated to alkaline phosphatase and antibodies bound to Rh polypeptides were detected by an alkaline phosphatase-conjugated substrate kit.

in a solid-phase ELISA with peptides coated on microtiter plates, by agglutination assays, and by Western blot with membrane proteins from RhD-positive (DccEE phenotype) RBCs. The results are summarized in Table 1.

Specificity of the polyclonal antipeptides antibodies. Solid-phase ELISA using 13 synthetic peptides (see Materials and Methods) coated to microtiter plates indicated that MPC4 and MPC6 reacted strongly with the immunizing peptides pRh8 and pRh6, respectively (not shown). No reactivity was observed with BSA or any other peptides. MPC4 reacted with peptide pRh8, but not with the pRh8₁ and pRh8₂ mutated peptides, which are specific for the Rh4 isoform of the CcEe transcript¹⁶ and the RhD protein,¹⁷ respectively, indicating that this antibody recognized an epitope expressed neither on RhD protein nor on certain isoforms of the CcEe proteins (Fig 1). MPC6 reacted strongly with peptide pRh3, which is present in CcEe protein isoforms,¹⁶ whereas MPC1 recognized the immunizing peptide pRh6 only when coupled to BSA, but reacted strongly in agglutination tests and Western blot analysis (see below). MPC8 reacted with the immunizing peptide pRh13, which is also present in CcEe protein isoforms and in the RhD polypeptide,¹⁷ but not with other peptides.

For comparison, the anti-Rh polypeptide raised in rabbits immunized with a partially purified RhD protein prepara-

tion²⁶ was tested with the 13 synthetic peptides and was found to react primarily in the ELISA test with peptide pRh4 (residues 185-202) located intracellularly within a predicted highly antigenic region (not shown). Moreover, the anti-Rh polypeptide did not recognize the pRh4₁ mutated peptide (see Materials and Methods), which is RhD-specific,¹⁷ although this antiserum reacted strongly with protein(s) from D--/D-- cells (see below). Further characterization of the antisera was performed by indirect agglutination of intact and enzyme-treated erythrocytes (papain, trypsin, or chymotrypsin). Only MPC1 reacted with RBCs from all common and rare variants tested (titer of 1:320), except for those from Rh_{null} and Rh_{mod} individuals. MPC4, MPC6, MPC8, and the anti-Rh protein neither agglutinated nor bound to intact RBCs (not shown), thus suggesting that the corresponding epitopes are either not exposed at the cell surface or may represent conformation-dependent structures.

Reactivity of rabbit antibodies with Rh proteins from common and variant RBCs. Membrane proteins from common and rare variant RBCs were separated on SDS-PAGE, transferred to nitrocellulose sheets, and probed by immunostaining using the rabbit antibodies (Fig 2). All antibodies reacted with a single band of Mr 32,000 clearly related to Rh proteins because no signal could be detected with mem-

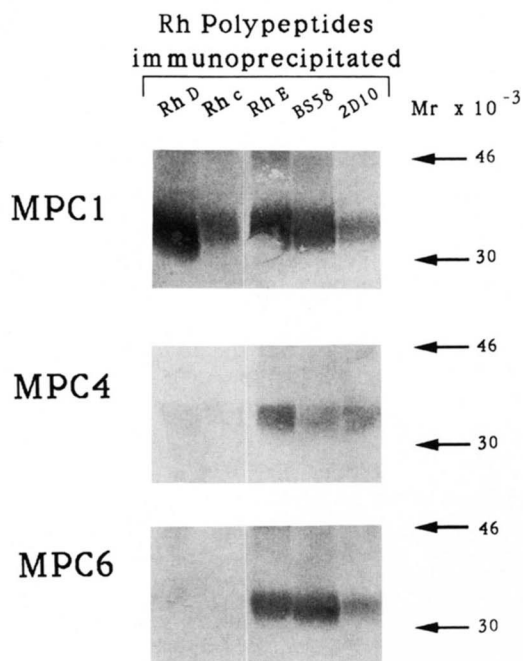


Fig 3. Staining of immunopurified Rh polypeptides with rabbit polyclonal antibodies. Immune complexes obtained from the reaction of human anti-D, -c, and -E, and monoclonal and murine 2D10 and BS58 MoAbs with Rh-positive cells (DccEE or DCcEe) were separated on SDS-PAGE (11% polyacrylamide gel) and transferred onto nitrocellulose sheets before incubation with MPC1 (1:4,000), MPC4 (1:800), and MPC6 (1:800) antibodies. After extensive washings, bound antibodies were detected as in Fig 2.

brane preparations from Rh_{null} or Rh_{mod} cells that either lack or are severely deficient in Rh proteins, respectively.

MPC1 (as well as MPC8, data not shown) and the anti-Rh polypeptide antibodies reacted with all membrane proteins tested, including the "deletion variants" (D-- and Dc-), D categories cells (D^{IV}, D^V and D^{VII}), and RhD-negative cells, but did not react with those from Rh_{null} and Rh_{mod}. Most likely, these antibodies should see distinct epitopes present on Rh peptides regardless of their blood group specificity. As the polyclonal anti-Rh polypeptide reacted on Western blot with Rh protein(s) from D--/D-- RBCs and this reactivity was not inhibited by the pRh4 peptide (data not shown), it is likely that this antiserum recognizes other peptides (D-specific?) not present in our panel.

MPC4 recognized the 32-Kd Rh proteins of dccEe, DccEE, and D^VcE RBCs, but reacted very weakly with the Rh proteins from the other common or rare RBCs that did not express either E and/or e antigens (Fig 2). Membrane proteins from homozygous RBCs with a partial (D--, D^{IV}C-, and Dc-) or total (Rh_{null} and Rh_{mod}) deletion of E and e antigens were completely unreactive with MPC4 and MPC6.

Reactivity of rabbit antibodies with immunopurified RhD, c, and E proteins. Immune complexes containing the Rh proteins were prepared by immunoprecipitation with human anti-Rh (anti-D, -c, and -E) and Rh-related

murine (2D10 and BS58) MoAbs, as previously described.^{20,21} Rh proteins dissociated from the complexes by heating in SDS were separated by SDS gel electrophoresis, transferred to nitrocellulose sheets, and probed with MPC1, MPC4, and MPC6 antibodies before incubation with a goat antirabbit IgG conjugated to alkaline phosphatase and color detection with a phosphatase substrate (Fig 3). MPC1 strongly stained Rh polypeptides immunoprecipitated with the different antibodies used. In contrast, MPC4 and MPC6 antibody stained most predominantly the RhE protein and only very weakly the RhD or Rhc proteins. The Rh-related polypeptides immunoprecipitated with BS58 and 2D10 were also stained by MPC4 and MPC6 (Fig 3), indicating that BS58 and 2D10 are reactive with cells carrying all Rh antigens, including Ee.

Reactivity of human and murine Rh and Rh-related antibodies with synthetic peptides. In parallel attempts to correlate peptide regions of the RhIXb protein with a given Rh specificity, different Rh-related murine MoAbs (R6A, 2D10, and BS58) and several human monoclonal and polyclonal anti-Rh antibodies (anti-D, -c, -C, -e, and -E) were tested in the ELISA assay with synthetic peptides coated to microtiter plates. The synthetic peptides were used either alone or tentatively as a mixture with peptide pRh10 that contains the unique exofacial Cys-284 critical for the Rh antigen reactivity.⁴ All reactions were negative, and no antigenicity could be restored in the presence of the pRh10 peptide.

Sidedness of antibody binding by Western blot analysis and ELISA. Because the C-terminus of the Rh proteins was predicted to be exposed extracellularly^{11,32,33} or intracellularly¹² in different reports, further studies were performed to address these issues by immunoadsorption of MPC1, MPC6, and MPC8 antibodies to ROVs or IOVs prepared from human erythrocytes. Unbound antibodies in supernatants and bound antibodies eluted from the vesicles were tested by Western blot and ELISA assays. As shown from Fig 4 and 5, only IOVs proved to be effective in binding MPC6 and MPC8, because these antibodies were detected in the acid eluates from IOVs but not from ROVs. Moreover, supernatants from IOVs contained only few residual antibodies, whereas those from ROVs reacted very strongly with the 32-Kd Rh proteins (Fig 4). None of the supernatants or acid eluates reacted with membrane proteins from Rh_{null} cells. In contrast, only ROVs were effective in binding MPC1, indicating that this antibody binds to a surface exposed epitope, as expected from agglutination studies showing that MPC1 reacted with RBCs from all common and rare variants (except Rh_{null}) in the antiglobulin test. As IOVs were slightly contaminated with ROVs, a faint staining in Western blot and a weak ELISA reactivity was noticed in the acid eluate from IOVs incubated with MPC1 (Figs 4 and 5).

Transient and stable expression of the RhIXb polypeptide in eukaryotic cells. Another attempt to establish the Rh specificity of the RhIXb-encoded protein was performed by eukaryotic expression of the RhIXb cDNA inserted in plasmid expression vectors (pcDNA1/NEO; Invitrogen Corp, San Diego, CA).³⁴ Transient and stable transformants of the

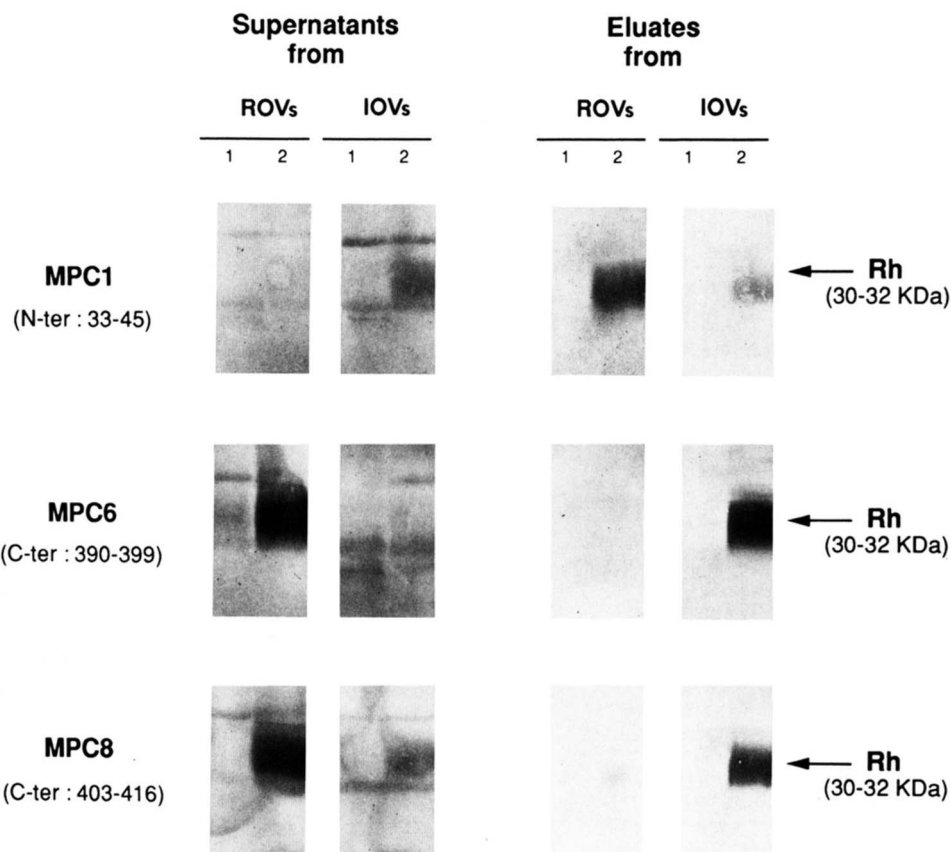


Fig 4. Analysis by Western blotting of antibodies in supernatants and eluates from ROVs and IOVs. Total membrane proteins from Rh_{null} (lanes 1) and DccEE (lanes 2) RBC membranes were separated on SDS-PAGE (12% polyacrylamide), electrophoretically blotted onto nitrocellulose, and immunologically stained with supernatants (1:500) or acid eluates (1:500) from IOVs and ROVs incubated with MPC1, MPC6, and MPC8 antibodies as described in Materials and Methods. Antibodies bound to Rh polypeptides (32 Kd) were visualized as described in Fig 2.

simian COS cell line and of the human HMy2.C1R monocytic cell line were analyzed by indirect fluorescence using antibodies against human (anti-D, -C, -c, -E, and -e) and rabbit (MPC1), and murine MoAbs (2D10, BS58, and R6A) that see epitopes normally exposed extracellularly on intact RBCs. No significant Rh expression could be detected either extracellularly or intracellularly on the transfected cell lines with these antibodies. Western blot analysis with MPC1 and anti-Rh rabbit antibodies that see denatured epitopes on Rh proteins blotted to nitrocellulose was also negative. Cotransfection into COS cells of the full-length RhIXb mRNA and its recently characterized¹⁶ alternative transcripts, either alone or together, also led to negative results (data not shown). Finally, attempts to express the RhIXb cDNA in stable transformants of human leukemic cell lines exhibiting erythroid characters (K562 and HEL) were similarly unsuccessful.

DISCUSSION

Four rabbit polyclonal antibodies (MPC1, MPC4, MPC6, and MPC8) raised against synthetic peptides (pRh6, pRh8, pRh3, and pRh13, respectively) derived from the predicted sequence of the protein encoded by the cDNA clone RhIXb have been characterized and used to investigate immunochemical properties of Rh proteins and to probe their topological organization within the cell membrane.

When tested in ELISA with 13 synthetic peptides derived from the Rh protein sequence (Fig 6), all antibodies reacted

only with the immunizing peptide, although MPC1 reacted only with peptide pRh6 coupled to BSA. In addition, MPC1 could agglutinate all human RBCs, except those from Rh_{null} individuals, but the other antibodies did not agglutinate native or enzyme-treated erythrocytes. This indicates that the pRh6 epitope (position 33-45) is probably conformation-dependent and located extracellularly. The anti-Rh protein produced by immunizing rabbits with a partially purified RhD protein preparation²⁶ investigated simultaneously was unable to agglutinate RBCs and was found to bind peptide pRh4 (position 185-202) predicted as intracellular.

All rabbit antibodies reacted on Western blot analysis with 32-Kd membrane proteins of human RBCs, except for those from Rh_{null} and Rh_{mod} individuals in which Rh proteins are lacking or severely reduced, respectively. MPC1 and MPC8 (and the rabbit anti-Rh protein) reacted with all membrane preparations, including those from RhD-negative individuals and those from common RhD-positive individuals as well as rare variants carrying no Cc and/or Ee antigens (D[—] and Dc[—]) or D categories cells (D^{IV}, D^V, or D^{VII}) that express only some of the D epitopes.³⁵ These data indicate that peptide pRh6 (residues 33-45) and pRh13 (residues 408-416) are common epitopes to all Rh proteins, regardless of their blood group specificity. In sharp contrast with the other antibodies, MPC4 reacted only in Western blot analysis with membrane preparations from cells carrying the E antigen, and MPC6 reacted only with cells carrying either the E or e antigens. Moreover, that the rabbit

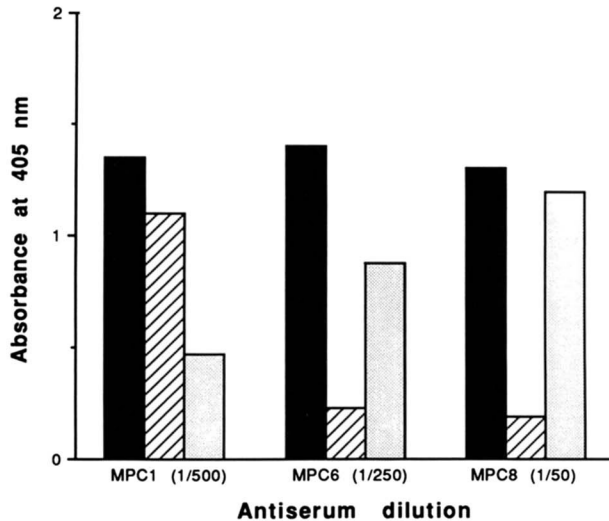


Fig 5. Analysis by solid-phase ELISA of antibodies in supernatants and eluates from ROVs and IOVs. Acid eluates from IOVs and ROVs incubated with MPC1, MPC6, and MPC8 were assayed by ELISA against 100 ng of specific peptide pRh6 coupled to BSA, pRh3, and pRh13, respectively, coated to the surface of a microtiter plate. The ordinate represents binding (mean of triplicate determinations) in relative units of optical reading at 405 nm. (■) Antiserum; (▨) eluate from ROVs; (□) eluate from IOVs.

antibodies reacted with authentic Rh proteins was confirmed by showing that these antibodies could stain on Western blot the Rh proteins immunoprecipitated with MoAbs such as anti-D, -c, and -E. Interestingly, MPC1 could blot to immunopurified D, c, and E proteins, whereas MPC4 and MPC6 blotted predominantly to E proteins and only very weakly to D or c proteins. The weak reactivity with D and c might result from trace contamination of E/e proteins in the D and c immune precipitates. Alternatively, a weak reactivity of MPC6 with D is rather expected because the amino acid region 390-404 of this protein (location of peptide pRh3) carries a Val → Glu mutation at position 397¹⁷ that

probably reduces or abolishes significant antibody binding. Similarly, there are two amino acid substitutions within the region 224-233 (location of pRh8) in the D protein that were shown to affect binding of MPC4, as found by no reactivity with the mutant peptides pRh8₁ and pRh8₂. On the other hand, the very weak reaction of MPC4 and MPC6 with protein c might result from cross-reactivity with an epitope shared by c and E/e proteins, but it could be decided only when the basis of the Cc/Ee polymorphism is understood. Altogether, these findings clearly show that MPC4 and MPC6 best recognize Rh proteins of E/e specificity and suggest that the polypeptide encoded by the RhIXb cDNA carries the E (or e) antigen. This finding correlates well with the recent demonstration by genomic DNA analysis that the RhIXb mRNA is transcribed from the *CcEe* gene.¹⁶ Although peptides pRh3 and pRh8 may be characteristic of RhE/e proteins, they are not recognized by human polyclonal antibodies or MoAbs that define E or e antigens on intact RBCs. This is not contradictory because it should be noted that the human anti-RhE/e antibodies do not react with denatured RhE/e proteins on Western blot, a further indication that Rh antigens are conformation-dependent. Data discussed in detail below strongly suggest that the pRh3 epitope detected by MPC6 is located intracellularly and is not available for binding to antibodies on intact cells. We assume, therefore, that the peptide sequence 224-233 (pRh8) might be of prime importance to determine E/e blood group specificity.

As the C-terminus of Rh proteins has been predicted to be exposed internally¹¹ or externally¹² in the membrane, the location of the pRh3 and pRh13 epitopes detected by antibodies MPC6 and MPC8 were examined by a method combining immunoadsorptions of these antibodies to ROVs and IOVs and analysis of the bound and unbound fractions by Western blot on membrane preparations from Rh-positive as well as Rh_{null} erythrocytes. When the MPC1 antibody was used as control in these experiments, it was found to bind ROVs but not IOVs, indicating the pRh6 epitope was exposed on an extracellular loop, as predicted from the to-

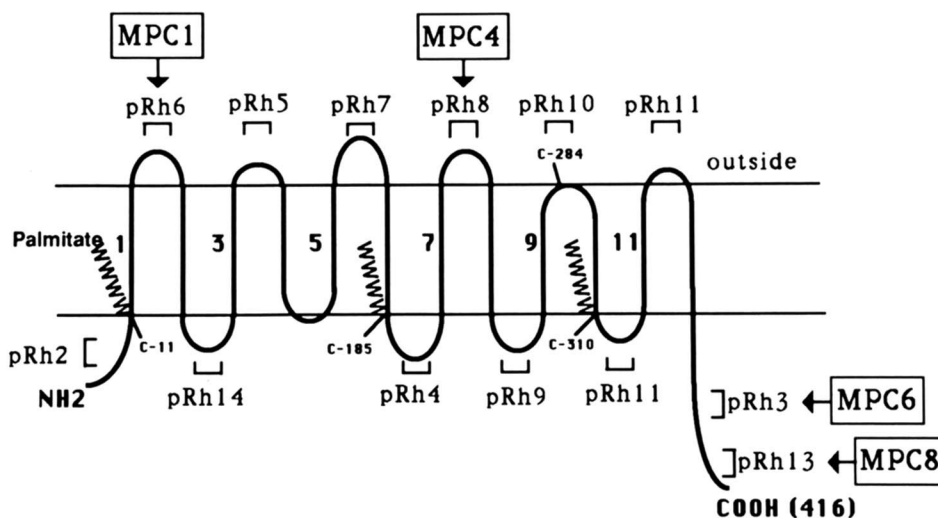


Fig 6. Proposed model of the Rh mature protein encoded by the RhIXb cDNA clone. Schematic drawing showing the topology of the Rh protein within the cell membrane, with both NH₂ and COOH termini located intracellularly. Regions corresponding to synthetic peptides used in this study are mentioned, as well as the polyclonal antibodies (boxes) specific for the indicated peptides. The surface exposed cysteine 284 involved in the immunoreactivity of Rh proteins and cysteines 11, 185, and 310 containing a potential palmitate fatty acid chain are indicated.

pologic model of Rh proteins^{11,12} and in accordance with the strong agglutination capacity of MPC1 (Table 1). MPC6 and MPC8, on the other hand, bound to the Rh polypeptides in its native conformation presented by IOVs only, indicating that the pRh3 and pRh13 epitopes located at the C-terminus of the Rh proteins were most likely located on the cytoplasmic side of the erythrocyte membrane (Fig 6). Surprisingly, this is in contrast with previous data^{11,32} that suggested the exofacial exposition of the C-terminus, as deduced from carboxypeptidase Y digestion of radioiodinated erythrocytes and derived vesicles³² and from membrane protein analysis of protease-digested intact erythrocytes by immunoblotting with anti-peptide antibodies directed against the N-terminus and the C-terminus of the cloned Rh polypeptide.³³ However, using a rabbit antiserum to the peptide residues 400-416 in a similar approach as developed in this study, Avent et al³⁶ also reported very recently that the C-terminal residues 400-416 of the Rh polypeptides are located intracellularly. Our present results further extend these data by showing that at least 27 residues (390 to 416) of the Rh proteins are apparently located inside the cell. The Rh proteins, therefore, should span the membrane 12 times (Fig 6). Although previous conclusions on the extracellular orientation of the C-terminus of Rh peptides might have resulted from a protease contaminant in carboxypeptidase Y preparations always difficult to avoid when high concentrations of enzyme are used,³² there are no clear explanations for discrepancy with the data reported by Suyama and Goldstein.³³ One possibility is that digestion of homozygous D— cells with phospholipase A2 followed by papain resulted in a partial hydrolysis only of the D polypeptide, which is plausible because the released C-terminal peptide was not monitored. Alternatively, one may speculate that the treatment by phospholipase A2 before papain digestion may alter the C-terminal orientation of the D protein or that the D protein itself of the D— cells has a different C-terminal orientation as compared with the D protein of common Rh phenotypes. Obviously, these points need further clarification before the membrane topology of Rh proteins can be conclusively established.

As a further approach to explore the structural and immunochemical properties of Rh proteins, attempts were developed to express Rh recombinant proteins into eukaryotic cells. In no instance could we find cell surface expression of Rh proteins in transient or stable transformants, as detected either by immunofluorescence analysis with a panel of MoAbs specific for polymorphic or nonpolymorphic epitopes or by Western blot analysis with the rabbit polyclonal antibodies. Transfection studies performed in erythroid cell lines (HEL and K562) were similarly unsuccessful. It is believed that these results support the concept that Rh antigen expression requires the correct transport and insertion of Rh proteins within the cell membranes, presumably in close association with one or several proteins (erythroid-specific?) that constitute the Rh protein complex and that is or are absent in Rh deficient erythrocytes.⁴ Coexpression of individual proteins of the Rh cluster in eukaryotic systems when the cDNAs become available may provide the key to understanding how Rh proteins are expressed in erythroid tissues.

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