Rh-RhAG/Ankyrin-R, a New Interaction Site between the Membrane Bilayer and the Red Cell Skeleton, Is Impaired by Rhnull-associated Mutation*

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Several studies suggest that the Rh complex represents a major interaction site between the membrane lipid bilayer and the red cell skeleton, but little is known about the molecular basis of this interaction. We report here that ankyrin-R is capable of interacting directly with the C-terminal cytoplasmic domain of Rh and RhAG polypeptides. We first show that the primary defect of ankyrin-R in normoblastosis (nb/nb) spherocytosis (HbH) is associated with a sharp reduction of RhAG and Rh polypeptides. Secondly, our flow cytometric analysis of the Triton X-100 extractability of recombinant fusion proteins expressed in erythroleukemic cell lines suggests that the C-terminal cytoplasmic domains of Rh and RhAG are sufficient to mediate interaction with the erythroid membrane skeleton. Using the yeast two-hybrid system, we demonstrate a direct interaction between the cytoplasmic tails of Rh and RhAG and the second repeat domain (D2) of ankyrin-R. This finding is supported by the demonstration that the substitution of Asp-399 in the cytoplasmic tail of RhAG, a mutation associated with the deficiency of the Rh complex in one Rhnull patient, totally impaired interaction with domain D2 of ankyrin-R. These results identify the Rh/RhAG-ankyrin complex as a new interaction site between the red cell membrane and the spectrin-based skeleton, the disruption of which might result in the stomato-spherocytosis typical of Rhnull red cells.

In red blood cells (RBCs),1 the membrane skeleton is a simple structure experimentally defined as the insoluble residue remaining after extraction of the red cell membrane with non-ionic detergents (1). The red cell skeleton is organized in a two-dimensional network predominantly composed of spectrin, actin, and protein 4.1, along with adaptor proteins like ankyrin, protein 4.2, p55, protein 4.9, adducin, tropomyosin, myosin, and tropomodulin (2). The integrity of this skeleton and its linkage with integral transmembrane proteins is crucial for the maintenance of the mechanical properties of the RBC. This is evidenced by the morphological abnormalities of the RBC that result in hemolytic anemia of varying severity (hereditary elliptocytosis (HE), pyropoikilocytosis (HPP), spherocytosis (HS), and ovalo-stomatocytosis (SAO)). These abnormalities are associated with mutations of different skeletal and integral membrane proteins that alter “horizontal” or “vertical” protein/protein interactions (3). The band 3-ankyrin protein 4.2 and the glycoporphin C (GPC) protein 4.1-p55 complexes represent well characterized major attachment sites between the membrane bilayer and the red cell skeleton (4, 5). However, there is increasing evidence, as discussed below, that another important interaction site involves the membrane protein complex that carry the Rh antigens.

The Rh blood group antigens are defined by a complex association of membrane polypeptides that includes the non-glycosylated Rh proteins (carriers of the RhD and RhCcEe blood group antigens) and RhAG, the Rh-associated glycoprotein, which is strictly required for cell surface expression of Rh (6, 7). Rh and RhAG are major components of the erythrocyte membrane (about 200,000 copies per cell) and exhibit structural homology with the NH4+ transporters of the Mep/Amt superfamily (8). Most importantly, recent independent studies have shown that RhAG and its kidney homologue RhCG can act as bidirectional ammonium transporters, at least when expressed in heterologous systems (9, 10). In red cells, a tetramer composed of two Rh and two RhAG subunits is thought to constitute the core of the Rh complex to which accessory chains (CD47, LW, and GPB) are associated by non-covalent linkages (11, 12). The morphological abnormalities of Rhnull erythrocytes (stomato-spherocytosis), associated with the primary defects of Rh or RhAG, suggest a biologically important association of the Rh complex to the red cell skeleton (11, 13). Supporting such a role, several studies indicate that Rh, RhAG, and CD47 are, in great part, resistant to membrane solubilization by non-ionic detergent and therefore remain predominantly associated with the detergent-insoluble material (DIM) of erythroid precursor cells cultured and differentiated in vitro, mature red cells, and erythroleukemic cell lines (14–16). Evidence that these results currently account for an association of the Rh complex with the erythrocyte membrane rather than with lipid rafts (defined as detergent insoluble lipid rich membrane microdomains) (17), has been recently provided by fluorescence imaged microdeformation (FIMD) analysis of intact RBCs (18). In these studies, the behavior of Rh and CD47 proteins were intermediate between that of actin and band 3, whereas that of RhAG was similar to that of GPC and actin (19,
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20). These results imply that the Rh complex, presumably via Rh and/or RhAG and/or CD47, is firmly linked to the spectrin-actin based erythroid membrane skeleton. However, which protein of the Rh complex and which skeletal protein are involved in this vertical interaction remain unknown. Recently, an association between CD47 and protein 4.2 was indicated by the observation that RBCs with complete protein 4.2 deficiency (4.2−/−IS) exhibit a 80–90% reduction of CD47 (21, 22). However, CD47 linkage to the red cell skeleton was found to be independent of Rh and RhAG (20), and neither the expression level nor the Triton X-100 extraction of Rh and RhAG from the cell membrane were altered by the absence of protein 4.2 in 4.2−/−/HbR.2 genotype. These results strongly suggest that protein 4.2 is not involved in linking the core of the Rh complex to the membrane skeleton.

Because protein 4.2 appears to interact with CD47 and has been experimentally shown to bind ankyrin (4), we hypothesize that Rh and RhAG, which are associated with CD47 within the Rh complex, might interact with the spectrin-based skeleton through an association with ankyrin. Ankyrins are adaptor proteins that constitute a family of proteins expressed in many cell types and possess binding sites for both integral and cytoskeletal membrane proteins. In vertebrates, three genes, Ank1, Ank2, and Ank3, control the tissue-specific expression of the ankyrin-R, ankyrin-B, and ankyrin-G proteins, respectively (23), and only ankyrin-R is expressed in erythroid cells. In this paper, we describe a direct and specific association between the C-terminal cytoplasmic domains of Rh and RhAG and the repeat domain D2 of the membrane binding domain of ankyrin-R. Furthermore, we demonstrate that a naturally occurring mutation of RhaG, associated with the stomato-spherocytosis phenotype of one Rhnull patient, has a deleterious effect on RhAG-ankyrin-R association. Therefore, we propose that Rh, RhAG, and ankyrin-R constitute a new major protein complex involved in the linkage of the red cell membrane lipid bilayer to the underlying skeleton.

MATERIALS AND METHODS

Materials—Monoclonal antibody (mAb) anti-Lu6 (clone LM342) was purchased from Dr. R. H. Fraser (Regional Donor Center, Glasgow, United Kingdom). Fluorescein isothiocyanate-conjugated F(ab′)2, phycoerythrin-conjugated F(ab′)2 fragments, and normal donkey serum were provided from Dr. R. H. Fraser (Regional Donor Center, Glasgow, United Kingdom). Primers used in polymerase chain reaction (PCR) were purchased from MWG Biotech (Ebersberg, Germany). The QuikChange site-directed mutagenesis kit and the Pfu Turbo DNA polymerase were provided by Stratagene. The pGEX-3X-5 vector, the glutathione-Sepharose 4B beads and [35S]methionine were purchased from Amersham Biosciences. The TNT T7/SP6-coupled reticulocyte lysate system were from Promega (Madison, WI). The pGBK7 and pGADT7 vectors from the Matchmaker Gal4 two-hybrid system III were provided by Clontech.

Analysis of Mouse RBC Membrane Proteins—Wild type (WBB6F1) and mutant mice were from The Jackson Laboratory. RBCs were collected from the intraorbital sinus of mice exhibiting selective phenotypes. Homozygous normohalostasis (ab+/ab) mice characterized by a complete deficiency in full-length 210-kDa ankyrin-R associated with spontaneous defect in the Ank1 gene were described previously (24). β-adducin−/− gene-targeted mice (25) were kindly provided by Dr. L. L. Peters (The Jackson Laboratory). Preparation of RBC membranes, SDS-PAGE, and Western blotting of membrane proteins were done as described previously (22). For flow cytometric measurement, 2 μl of packed red cells were fixed at room temperature in 1 ml of 1% formaldehyde for 15 min and then washed three times with binding buffer and once with binding buffer containing 0.2% bovine serum albumin and incubated with a donkey serum for 20 min at room temperature to eliminate unspecific signals. Following incubations with mAbs anti-Rh (26) or the mCTRHaG anti-mouse RhAG (7) polyclonal antibodies, cells were incubated with a donkey anti-rabbit phycoerythrin-conjugated antibody and analyzed using a FACSCalibur™ flow cytometer (BD Biosciences). Stainings with the rat anti-murine CD47 (27) or the human anti-Di (HIRO-71) and anti-Di (HIRO-58) (generous gifts from Dr. Uchikawa, Red Cross of Japan), mAbs were carried out similarly except that the cells were not fixed or permeabilized. Immunoperoxidase or anti-human phycoerythrin-conjugated antibodies were used. As expected from comparative analysis of mouse band 3 cDNA sequence (28) and the amino acid polymorphism associated with the Di/Di blood group polymorphism in man (P854L) (29), mouse band 3 could be detected by the anti-Di but not the anti-Di mAb. To check the permeabilization efficiency, actin staining was performed on all cells at room temperature with 10 μl of phalloidin-fluorescein isothiocyanate (Sigma).

Lu Fusion Protein Constructs, Transfection, and Flow Cytometric Analysis—A Lu-tailless expression vector was constructed by in vitro mutagenesis of the pDNA3-Lu6 plasmid described previously (30). An EcoRI subcloning site and a following TAA stop codon were introduced at position 1720 (+1 taken as the first nucleotide of the initiator ATG codon). Sequences for the 29, 26, 47, and 28 amino acid-long cytoplasmic C-terminal tails of Rh, RhAG, and the Duffy antigen receptor for chemokines (DARC), respectively, were obtained by PCR. Lu-Rh, Lu-RhAG, Lu-GPC, Lu-DARC fusion proteins were constructed by introducing the PCR products at the EcoRI site of the Lu-tailless construct. Human erythroleukemia K562 cells, obtained from the American Type Culture Collection, were stably transfected by the Lu fusion plasmids and grown in RPMI 1640 and 10% fetal calf serum in the presence of 0.6 g/liter neomycin (G418). Lu-positive cells were detected by flow cytometry using the anti-Lu6 mAb. The linkage between each fusion protein and the DIM was assessed by comparing the anti-Lu6 antibody binding capacity of intact and Triton X-100 treated cells, as described previously (16).

Yeast Two-hybrid Studies—The D1, D2, D3, and D4 subdomains of the membrane binding domain of ankyrin-R were amplified by PCR using previously described plasmids as templates (31). The four domains were inserted between the EcoRI and BamHI sites of pGADT7 in-frame with the GAL4 activation domain. cDNA fragments encoding the C-terminal domains of Rh and RhAG were amplified by PCR and inserted into pGBK7 to generate fusion genes with the DNA binding domain of GAL4. Mutant forms of RhaG were obtained by site-directed mutagenesis. The pGBK7 and pGADT7 constructs were co-transformed into the AH109 yeast strain using the Li acetate method (32). The AH109 cotransformants were grown on plates lacking leucine and tryptophane. After 3 days of growth at 30°C, the colonies were streaked onto plates containing histidine, adenine, leucine, and tryptophane. Protein interactions were analyzed 3 days following plating and confirmed by β-galactosidase assays as recommended by the manufacturer (Matchmaker Gal4 two-hybrid System III, Clontech).

GST Pull-down Assays—The PCR amplified cDNA fragment encoding the C-terminal end of RhAG was fused in-frame with the glutathione-S-transferase (GST) gene. The GST fusion proteins expressed in Echerichia coli BL21 were purified by elution from glutathione-Sepharose beads. The PCR-amplified D2 subdomain of ankyrin-R was subcloned into the BglII-SpeI restriction sites of the in vitro expression vector pT7T7 and in vitro translated using the TNT T7/SP6-coupled reticulocyte lysate system in the presence of [35S]methionine. For the GST pull-down assay, 7.5 μg of GST fusion proteins were bound to 50 μl of glutathione-Sepharose beads in PBS containing 1 mM dithiothreitol. Nonspecific protein binding sites were blocked by incubation for 20 min at 4°C with 2% bovine serum albumin. After two washes with binding buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20), equal amounts of in vitro translated protein were incubated for 4°C with binding buffer lacking Tween 20. Proteins were then eluted with 35 μl of glutathione buffer, and binding reactions were analyzed by autoradiography of 12.5% SDS-PAGE gels.

4 N. Mohandas and J. P. Cartron, unpublished results.

5 Y. Nicolas, C. Le Van Kim, P. Gane, C. Birkenmeier, J. P. Cartron, Y. Colin, and I. Mouro-Chanteloup, unpublished results.
Experiments were performed on RBCs from three mice of each phenotype. Results on mCTRhAG pAbs and phalloidin, respectively. CD47 and band 3 were detected on intact cells using the iap301 and HIRO-58 mAbs, respectively. "Expression of murine Rh, RhAG, and actin was analyzed on permeabilized RBCs, as described under Materials and Methods," using the MPC8 and mCTRhAG pAbs and phalloidin, respectively. CD47 and band 3 were detected on intact cells using the iap301 and HIRO-58 mAbs, respectively. Results on nbnb and adducin−/− RBCs are expressed as percentages of the mean fluorescence intensity (±S.E.) obtained with wild type (wt) RBCs. Experiments were performed on RBCs from three mice of each phenotype. N.D., not determined.

RESULTS

Ankyrin-R-deficient Mouse RBCs Are Rh- and RhAG-deficient—Cell surface expression of the Rh complex on RBCs from ankyrin-R-deficient (nbnb) and β-adducin-deficient (adducin−/−) mice was compared with wild type by flow cytometric analysis of Rh, RhAG, and CD47, as described previously (22). This analysis revealed that Rh and RhAG were reduced by 53 and 97%, respectively, in RBCs from nbnb mice (n = 3) compared with wild type and adducin−/−, whereas CD47 was normally expressed in all samples (Fig. 1). To validate these results, two controls were performed. i) Cell surface expression of murine band 3 was found to be reduced by 50% in n/nb mice, which is in agreement with previous chromatographic analysis of n/nb RBC membrane proteins (33). ii) The fluorescence given by the binding of phalloidin to actin was analyzed on intact and permeabilized/fixed RBCs, as expected, no signal was obtained in non-treated RBCs (not shown), whereas similar mean fluorescence intensity (MFI) values were obtained with all permeabilized RBCs (Fig. 1), indicating that the reduced reactivity in n/bnb RBCs of antibodies against intracellular epitopes of Rh and RhAG could not result from a potentially reduced permeabilization efficiency of these cells associated with their membrane abnormalities.

Immunoblotting experiments gave results that support the flow cytometric analysis with a deficiency of Rh and RhAG in n/bnb RBC membrane protein preparations (Fig. 2). It is noteworthy that the reduction of Rh was much more drastic than would be expected from the 50–60% reduction deduced from flow cytometric analysis and that the amount of CD47 appeared reduced in n/nb red cell membrane sample despite a normal cell surface expression (Fig. 1). This illustrates, as was pointed out previously in the course of band 3−/− mouse RBC analysis (34), the difficulty of quantitatively comparing normal and mutant membrane proteins by SDS-PAGE and immunoblotting experiments because of the excessive hemolysis, hemoglobin-membrane association, and partial protein degradation in HS mouse samples. However, comparison of the ratio between CD47 and Rh or RhAG in wild type and n/nb samples (Fig. 2) clearly confirmed the deficiency of Rh and RhAG in n/nb mouse RBCs.

Fusion Proteins Containing the C-terminal Cytoplasmic Domains of Rh and RhAG Can Interact with DIM of Transfected K562 Erythroleukemic Cells—We have previously developed a flow cytometry-based method to analyze the association of transmembrane proteins with the erythroid skeleton as defined by the DIM obtained after Triton X-100 extraction of the lipid bilayer from K562 erythroleukemic cells. Using this approach, we have estimated that 54 and 79% of Rh and RhAG molecules were DIM-associated in K562 cells, respectively (16). To evaluate the specific role of the C-terminal tails of the 12 transmembrane domain (TM) Rh and RhAG proteins in the association with the DIM of K562 cells, we have constructed fusion proteins in which the 29 and 26 C-terminal residues of Rh and RhAG, respectively, were directly fused to the single extracellular and transmembrane domains of the Lu adhesion molecule (Fig. 3A). As shown in Fig. 3B, comparison of the anti-Lu antibody binding capacity of intact and Triton X-100 treated...
cells revealed that 59 and 65% of Lu-Rh and Lu-RhAG, respectively, were linked to the DIM of transfected K562 cells. As positive controls, 50% of the intact Lu glycoprotein and 68% of the Lu-GPC fusion protein in which the cytoplasmic tail of Lu has been replaced by that of GPC remained attached to the DIM. As negative controls, the truncation of the cytoplasmic tail of Lu (Lu-tailless protein) or its replacement by the C-terminal cytoplasmic domain of DARC (Lu-DARC fusion protein), a seven TM domain chemokine receptor not associated with DIM (16), resulted in a significant decrease of DIM association (±S.E.) to the DIM is indicated for each fusion protein. Values are from at least four independent experiments.

The C-terminal Cytoplasmic Domains of Rh and RhAG Bind to the D2 Repeat Domain of the Membrane Binding Domain of Ankyrin-R—Considering the results presented above, we used the yeast two-hybrid assay to test the hypothesis that the C-cytoplasmic tails of Rh and RhAG might interact with ankyrin-R and particularly with one or several of the four N-terminal ankyrin repeat domains shown previously to interact with various transmembrane proteins. The C-terminal domains of Rh and RhAG were fused in-frame to the GAL4 DNA binding domain of the yeast two-hybrid pGBK7 vector, and the four repeat domains of ankyrin-R (D1, D2, D3, and D4) were individually fused in-frame with the GAL4 activation domain of the pGBK7 vector. The recombinant vectors were cotransformed in yeast, and their ability to grow in high stringency selective media lacking adenine, histidine, leucine, and tryptophane was analyzed (Fig. 4, A and B). These experiments, confirmed by β-galactosidase activity analysis (not shown), indicated that the C-terminal cytoplasmic domains of Rh and RhAG specifically interact with the D2 repeat domain of ankyrin-R. No association between the cytoplasmic tails of Rh and RhAG and the remaining D1, D3, and D4 repeat domains of ankyrin could be detected (Fig. 4) even when yeasts
were grown in less stringent medium lacking histidine, leucine, and tryptophane but containing adenine (data not shown). A survey of the literature revealed that one missense mutation (H276R) in the D2 domain is associated with the HS phenotype of one Brazilian patient (35). However, as analyzed by two-hybrid assay, this mutation has no effect on Rh/RhAG-AnkR interaction (not shown).

**The Rhnull-associated Substitution of Asp-399 Alters Interaction of RhAG to Ankyrin-R and to DIM of K562 cells**—Mutation of Asp-399 has been identified previously in the cytoplasmic tail of RhAG from one Rhnull patient (11). We have now generated mutated forms of RhAG in which Asp-399 or the closely located Asp-398 and Asp-397 residues have been replaced by alanine and tested their capacity to bind to the D2 domain of ankyrin using the two-hybrid system described above. Neither the D399A nor the D398A substitutions altered the growth of RhAG, as assessed by Coomassie staining (Fig. 5A) or its β-galactosidase activity (not shown). In contrast, substitution of Asp-399 abolished both the capacity of cotransformed yeast to grow on restrictive media lacking adenine, histidine, leucine, and tryptophane indicating a specific interaction of Rh and RhAG with domain D2 of ankyrin-R. The previously described interaction between the large T-antigen of SV40 (T-Ag) and murine p53 (44) was used as positive control. As negative controls, Rh- and RhAG-pGBK7 vectors were cotransformed with the T-Antigen-pGADT7 plasmid and the Ank-D-pGADT7 vectors were cotransformed with the p53-pGBK7 plasmid. For each co-transformation, three colonies were streaked on selective media. Ank-D* is for Ank-D1, Ank-D2, Ank-D3, or Ank-D4.

A powerful and simple approach to identify potential interactions between skeletal and integral membrane proteins is to compare the expression of cell surface markers in abnormal RBCs with well defined deficiency of skeletal proteins to that of normal RBCs. Because Rh and RhAG deficiency is associated with stomato-spherocytosis of Rhnull red cells, we and others have analyzed Rh complex expression in some HS red cells with primary defects in different skeletal or transmembrane proteins. Using this approach, a positive correlation between the expression levels of CD47 and protein 4.2 has been recently reported from the analysis of 4.2(HS) red cells (21, 22). However, as discussed in our introduction, the lack of protein 4.2 has no deleterious effect on the expression level and skeleton linkage of Rh and RhAG. These observations indicate that defective interaction(s) between CD47 and protein 4.2 could not be responsible for cell shape abnormalities of Rhnull red cells and that other proteins must be involved in the interaction of the Rh complex to the skeleton. Band 3, the red cell anion exchanger, has recently been proposed to represent such a protein. Indeed, from the observation that expression of the Rh complex is sharply decreased in human and mouse HS RBCs with a primary defect in band 3, it has been postulated that Rh and RhAG, as part of a potential band 3-based macrocomplex,
might be indirectly linked to the red cell skeleton through direct lateral association with band 3 (36). However, Rh and RhAG are associated with the skeleton of K562 cells and early erythroid precursors (16) that lack band 3 to the same extent as with the skeleton of mature red cells that possess band 3 (19, 20). These results indicate that the core of the Rh complex might interact with the erythroid skeleton regardless of the presence or absence of band 3 and, therefore, do not support a model in which a direct interaction between band 3 and Rh/RhAG could modulate the association of the Rh complex to the skeleton.

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HS red cells from ankyrin-deficient normoblastosis mice (nb/nb), indicates ankyrin-R as a potential binding partner of the Rh complex. It is noteworthy that the cytoplasmic tails of Rh and RhAG are particularly well conserved across species as compared with the other domains of these 12 TM proteins (37) and are capable of mediating the attachment of Lu-fusion transmembrane proteins to the cytoskeleton of K562 cells to the same extent as the whole Rh and RhAG proteins (our present results and Ref. 16). We therefore assume that interaction between these domains and ankyrin-R plays a major role in the attachment of Rh and RhAG to the red cell skeleton. Supporting this hypothesis, the major finding of this study is the demonstration of a direct interaction in vitro between the cytoplasmic tails of human Rh and RhAG proteins and the D2 repeat subdomain of the membrane binding domain of ankyrin-R. The D2 domain of ankyrin-R was previously shown to mediate attachment of the spectrin-based membrane skeleton to a diverse set of membrane-spanning proteins like CD44 (38) and band 3 (39). A previous comparison between binding sites on ankyrin for neurofascin and the anion exchanger (31) demonstrated that both proteins can interact simultaneously with ankyrin. Ankyrin can also form homocomplexes between two band 3 dimers via two distinct but cooperative binding sites (D2 and D3-D4) (39). Consistent with this property of ankyrin to form homo- and heterocomplexes between membrane proteins, we propose a model for band 3-ankyrin-R/RhAG interactions in which ankyrin-R can bind directly and simultaneously to band 3 and Rh and/or RhAG via its D3-D4 and/or its D2 repeat domains respectively (Fig. 6). In agreement with this model, Rh and RhAG expression could be reduced not only in HS red cells with a primary defect of ankyrin, as in nb/nb mice (our present results), but also in HS red cells with a defect of ankyrin secondary to a primary defect in other membrane component, like band 3 (36). Hence, we assume that the reduced expression of Rh and RhAG in mouse and human band 3 null HS red cells might result from the secondary 60% reduction of ankyrin-R that is observed in these cells (34, 40, 41). Conversely, we postulate that the enhancing effect of band 3 on Rhnull cells and that these cells contain a normal amount of Rh/RhAG-ankyrin R membrane complex and its potential involvement in the morphological properties of the RBCs. Because Rh proteins and ankyrin-R have isoforms or homologues in nonerythroid cells, it is assumed that the Rh/RhAG-ankyrin interaction described in RBCs may also exist in other cell types. Further studies will be necessary to characterize these interactions in nonerythroid cells and determine whether and how they could modulate the emerging transporter function of different members of the Rh protein family.

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