Structure and Expression of the RH Locus in the Rh-Deficiency Syndrome


Red blood cell deficiency of Rh proteins is associated with morphologic and functional abnormalities of erythrocytes and with a chronic hemolytic anemia of varying severity. Rh-deficiency may be the result of homozygosity either for a silent allele at the RH locus (Rhnull amorph type) or for a recessive inhibitor gene(s) at an autosomal locus unlinked to RH locus (Rhnull regulator and Rhmod). In this report, we investigated the RH locus structure of Rh-deficient individuals by Southern analysis using cDNA and exon-specific probes deduced from the recent cloning of RH genes (CeEe and D). As expected from family studies indicating that Rhnull and Rhnull regulator individuals are unable to express Rh antigens but are able to convey functional RH genes from one generation to another, no alteration of the RH genes was detected in these variants. Although Rhnull of the amorph type arose by inheritance of a pair of silent alleles at the RH locus, the general organization of the RH locus was not altered. The amorph type may arise from two distinct genetic backgrounds. The most common type of Rhnull, called the “regulator type” occurs by an inhibition mechanism. According to Levine et al., this phenotype is caused by homozygosity for an autosomal recessive suppressor gene (Xr) that is genetically independent of the RH locus. The second type of Rhnull, which was first noted in a Japanese family, is called “amorph” or “silent type” and arose by homozygosity for a silent allele at the RH locus. The Rhmod phenotype was discovered more recently and was found to arise from the homozygous state of a suppressor gene (Xr) at an autosomal locus independent of RH. However, the Xr and Xs nomenclature is inappropriate, because these genes are not located on the X chromosome and because it is not known whether they are alleles at the same locus. The amorph type of Rhnull is much less common than other Rh-deficient types. Of 42 recently listed Rh-deficient phenotypes, only 5 are the amorph type of Rhnull. All types of Rh deficiency show the same clinical abnormalities associated with a chronic hemolytic anemia, stomatocytosis and spherocytosis, reduced osmotic fragility, and increased cation permeability. In addition, Rhnull membranes have characteristically hyperactive membrane adenosine triphosphatases (ATPases), reduced RBC cation and water contents, and a relative deficiency of membrane cholesterol. Rhnull cells also lack several membrane proteins and glycoproteins (GPs), including the 30- to 32-Kd proteins carrying the Rh antigens and the Rh-related GPs recognized by murine monoclonal antibodies. In addition, glycoporphin B, the carrier of S antigens, is reduced to approximately 30% of the normal level; certain other blood group antigens, including LW, Duffy (Fy5), U, and Duclos antigens, are also missing or severely decreased in these cells. One important and still unresolved question is to explain how a single genetic alteration at the RH locus itself or at an independent locus controlling its expression (Xr, Xs) may result in so many membrane abnormalities. To address this issue we have examined the structure and expression of the RH locus in these patients using the recently available information deduced from the cloning of the two RH genes that compose the RH locus.

MATERIALS AND METHODS

Patients. Blood samples from Rh-deficient patients were collected on heparin (10 U/ml) and shipped to Paris. Three samples of Rhnull of the regulator type were investigated: A.L., provided by L. Lebeck (Loma Linda, CA); Y.T., provided by C. Hyland (Brisbane, Australia); and T.B., provided by Dr. M.J. Stelling (Geneva, Switzerland). One sample of Rhnull of the amorph type (D.A.A.) was from Dr. C. Perez-Perez (Linarees, Spain) and one sample of Rhmod (S.S.) from P. Hartnett (Miami, FL). Blood samples from individuals with common Rh phenotypes were from the National Blood Transfusion Service in Paris.
Fig 1. Immunostaining of RBC membrane proteins from Rh-deficient erythrocytes. One hundred to 150 μg of total membrane proteins from A.L. (Rh+, reg, U-negative), S.S. (Rh+, U-negative), and D.A.A. (Rh+, silent, U-positive) separated by SDS-PAGE on a 12% polyacrylamide separating gel were transferred to nitrocellulose sheets. Each was incubated with rabbit anti-Rh protein antibodies (MPC1 and MPC8, 1:4,000 dilution), with a murine MoAb anti-LW (BS46; 10 μg/mL), with the murine MoAb 2D10 (10 μg/mL), and with the rabbit antiprotein 7.2b antiserum, respectively. Antibodies bound were detected by the alkaline phosphatase conjugate substrate method. Arrows on the left side indicate the migration position of the Rh, LW, 2D10, and 7.2b polypeptides.

Membrane protein analysis. For immunostaining analysis, sodium dodecyl sulfate (SDS)-lysates from RBC membrane preparations prepared as described were separated by discontinuous SDS polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose sheets, and incubated as described with rabbit polyclonal antibodies raised against synthetic Rh peptides (MPC1 and MPC8 reacting with the NH2- and COOH-terminal regions of the Rh proteins, respectively) or with the murine MoAbs BS46 (gift of Dr H.H. Sonneborn, Dreieich, Germany) and 2D10 (gift of Dr A.E.G. von dem Bome, Amsterdam, The Netherlands) that react with blood group LW or Rh-related membrane GPs, respectively. A rabbit polyclonal antibody against the membrane protein 7.2b (gift of Dr R. Johnson, Detroit, MI) was also used as control. Bound antibodies were detected with alkaline phosphatase-labeled goat antirabbit IgG or rabbit antimouse diluted (1:800) followed by revelation with the alkaline-phosphatase conjugate substrate kit (Biorad Laboratories, Rockville Centre, NY).

Genomic DNA analysis. Human leukocyte DNA extracted from peripheral leukocytes was digested with restriction enzymes (Appligene, Strasbourg, France; 10 U/mg DNA), resolved by electrophoresis in 0.8% agarose gel, and transferred onto nitrocellulose membrane (BA85; Schleicher & Schüll, Dassel, Germany) as described. DNA probes used for hybridizations were: (1) the complete RhXb cDNA encoding the Cc/Ee proteins, (2) exon-specific sequences obtained by PCR from exon 1, exon 4, and exon 9 + 10 of the CcEe gene (Cherif-Zahar et al, unpublished data). Hybridization with DNA probes (10⁶ cpm/mL) was performed for 16 hours at 65°C in 5 × SSPE (1 × SSPE = 0.15 mol/L NaCl, 0.01 mol/L Na₂HPO₄, 0.001 mol/L EDTA, pH 7.4), 5 × Denhardt (1 × Denhardt = 0.02% wt/vol each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin [BSA]), 0.1% SDS, and 100 mg/mL sonicated salmon-sperm DNA. Final washes were performed at 65°C for 15 minutes in 0.1 × SSPE, 0.1% SDS.

Reverse transcription coupled with PCR amplification. Total RNA was extracted by the acid–phenol-guanidinium method from 5 mL of peripheral blood (PB) and 0.5 μg was incubated for 40 minutes at 42°C in a reaction mixture (50 μL) containing 100 mmol/L Tris (pH 8.3), 140 mmol/L KCl, 10 mmol/L MgCl₂, 30 mmol/L β-mercaptoethanol, 1 mmol/L of each deoxynucleoside triphosphate (dNTP), 40 U of ribonuclease inhibitor (RNasine),
Fig 2. Southern blot analysis of the RH locus in the Rh-deficient patients. DNA from RhD-positive and RhD-negative donors with the indicated phenotype and from Rh-deficient individuals were digested by HindIII or BamHI restriction enzyme and hybridized on Southern blot with the exon-specific Rh probes (A) as indicated or (B) with the full-length Rh cDNA probe.

and 10 U of avian myeloblastosis virus reverse transcriptase (Promega Biotec, Madison, WI). The cDNA products were then subjected to PCR in 50 mmol/L KCl, 10 mmol/L Tris (pH 8.3), 0.01% gelatin, 0.2 mmol/L of the four dNTPs, 50 pmol of each primer, and 2.5 U of Thermus aquaticus DNA polymerase (Perkin-Elmer, Norwalk, CT). All primers were chosen from the RhXb cDNA sequence. PCR amplifications (30 cycles) were performed under the following conditions: 1 minute of denaturation at 94°C, 1.5 minutes of primer annealing at 55°C, and 2 minutes of extension at 72°C. Amplification products were purified using agarose gels electrophoresis, phosphorylated with polynucleotide kinase, and subcloned in pUC 18 vector.

DNA sequencing. Inserts from recombinant pUC18 vectors were sequenced on both strands using the dideoxy chain termination method.34

RESULTS AND DISCUSSION

Protein defects. In preliminary studies membrane preparations from the regulator (reg), the amorph (silent), and the Rhnull types of Rh-deficient erythrocytes that are unreactive (or very weak reactive for Rhnull) with all anti-Rh antibodies of different specificities (D, C, e, etc) were separated by SDS-PAGE and examined by immunostaining with antibodies raised against synthetic peptides and directed against the Rh proteins (30 to 32 Kd), the LW GP (42 Kd), and Rh-related GPs (45 to 75 Kd). Neither the MPC1 nor the MPC8 antibodies that recognize the NH2-terminal (residues 34 to 46) and the COOH-terminal (residues 409 to 417) of the Rh proteins,26,30 respectively, from RhD-positive (DCCee) and RhD-negative (ddcee) samples, reacted with the membrane proteins of Rh-deficient cells (Fig 1). Because the Cc/Ee and D proteins have conserved NH2- and COOH-terminal domains,28 it is concluded that the Rh-deficient cells lack both the Cc/Ee and the D proteins. This conclusion is consistent with the finding that the polyclonal antipeptide antibodies react with RBCs from homozygous D-- individuals who carry the D but not the Cc and Ee proteins.30 The present immunoblotting analysis of Rh-deficient cells, together with those reported by Suyama and Goldstein,35 support the conclusions of previous studies that have suggested the lack of Rh proteins in Rhnull cells using the indirect method of labeling.13,14 Although Rh-deficient erythrocytes and RBCs from patients with hereditary stomatocytosis show similar morphologic abnormalities, both conditions are distinct at the molecular level because the antiprotein 7.2b antiserum that selectively recognized a 31-Kd protein that was deficient in patients with hereditary stomatocytosis37 reacted normally with all Rh-deficient erythrocytes (Fig 1).

Rh-deficient cells also lack the LW and Rh-related GPs.15,26,38 The BS46 (anti-LW) antibody reacted only with RBC membrane proteins from common Rh phenotypes and not with those from Rh-deficient individuals (Fig 1). The murine MoAb 2D10 also recognizes membrane GPs of 45 to 75 Kd that are absent from most Rhnull or Rhmed cells lacking the blood group U, but are weakly expressed on some Rhnull cells that are U-positive.18,19 Accordingly, 2D10 did not react with the U-negative A.L. and S.S. samples (Fig 1) but did react weakly with the U-positive D.A.A. sample.26 These multiple protein abnormalities have not been explained on a molecular basis. A currently favored hypothesis is that Rh polypeptides are required for the correct transport or cell-surface expression of certain GPs or, alternatively but not exclusively, that a GP missing in Rhnull cells is necessary for the cell-surface expression of Rh pro-
the RH locus is composed of two homologous genes (ccddee sample, as expected from recent studies showing that exon-I: 2.5 kb; exon-4: 7.3 kb exon-9+10: 18 kb) with the sample (in the HindIII profile, ie, exon-I: 2.7 kb and 2.5 kb Two hybridizing bands were detected with each exon-specific gene.

Southern blot analysis of the RH locus in Rhnull and Rhmod phenotypes. Genomic DNA from the Rhnull reg Y.T., the Rhnull silent D.A.A., and the Rhmod S.S. were digested with HindIII and BamHI restriction enzymes and subjected to Southern blot analysis with Rh-specific probes.DNAs from RhD-positive (DCCee) and RhD-negative (ddccee) donors were used as a control. Figure 2A shows the hybridization patterns obtained with probes for exon-1, exon-4, and exon-9+10 of the CcEe gene (Cherif-Zahar et al, unpublished data) that correspond to residues (1-48, 162-211, and 408-416, respectively) of the mature CcEe-encoded proteins. Two hybridizing bands were detected with each exon-specific probe after HindIII and BamHI digestion of the DCCee sample (in the HindIII profile, ie, exon-1: 2.7 kb and 2.5 kb; exon-4: 7.3 kb and 6.7 kb; exon-9+10: 21 kb and 18 kb). However, only one band was detected (HindIII profile, exon-1: 2.5 kb; exon-4: 7.3 kb; exon-9+10: 18 kb) with the ccddee sample, as expected from recent studies showing that the RH locus is composed of two homologous genes (D and CcEe) in RhD-positive individuals, but of only one (CcEe) in RhD-negative individuals.24

The hybridization patterns of Y.T. (Rhnull reg) and S.S. (Rhmod) did not differ from those obtained with the RhD-positive control, and those from D.A.A. (Rhnull silent) were identical with those of the control RhD-negative phenotype (Fig 2A). No additional or missing band could be detected with the Rh-deficient samples suggesting a large sequence insertion and/or deletion at the RH locus of these individuals. This inference was confirmed by hybridization with the full length RhlXb cDNA probe (Fig 2B). It is not surprising that no defect of the RH genes occurs in Rh deficiency caused by a postulated recessive suppressor gene independent of the RH locus. Studies of families have shown that these individual family members carry the normal Rh genes but fail to express Rh antigens.3 Our results provide the first direct proof that confirms this hypothesis.

Hybridization with exon-specific probes was also performed to determine whether the Rh-deficient individuals carrying the D gene were homozygous or heterozygous for this gene. Gene dosage effects were obvious when HindIII digests of DNA from individuals of known DD, Dd, and dd genotypes, which all carry two copies of the CcEe gene, were estimated by densitometric analysis of autoradiograms (Fig 3). As expected from the presence of two copies of the D and CcEe genes in DD genome, the D-specific and CcEe-specific fragments24 were detected with the same intensity as with the exon 4 probe (6.7 kb and 7.3 kb, respectively) and the exon 1 probe (2.7 kb and 2.5 kb, respectively). On the other hand, a 1:2 gene dosage effect was observed between the D and CcEe fragments in the heterozygous Dd genome. Only the CcEe gene was detected in the Rh-negative (dd) DNA. Examination of the hybridization pattern of the Rh-deficient samples (Fig 3) showed that S.S. (Rhmod) and Y.T. (Rhnull reg) were homozygous for the D gene, whereas A.L. (Rhnull reg) was heterozygous for the D gene. T.B. (Rhnull reg) and D.A.A. (Rhnull silent) carried only the CcEe gene as Rh-negative donors. Although the Rh genotypes of the Rh-deficient individuals in this study were not known, these findings agree with studies of families that suggest that Rh haplotypes carrying or not carrying the D gene are segregating in Rhnull reg and Rhmod pedigrees.39,40 These data also show that the regulatory genes that determine the Rhnull reg and Rhmod phenotypes are effective both on chromosomes that carry either one or two Rh genes (CcEe or CcEe and D). Moreover, our findings show for the first time that zygosity for the D gene (DD or Dd) can be easily determined without studying families by using exon-specific probes on a DNA sample. Together with the possibility of detecting the D gene,23 this may have applications, for instance, in the prediction of the D genotype of a father in couples where there is an RhD-negative woman at risk of fetal alloimmunization.41

Southern blot analysis of the RH locus in the Rhnull silent family. Genomic DNA isolated from Epstein-Barr virus (EBV)-immortalized lymphoblastoid cell lines derived from each family member of the Rhnull silent D.A.A.28 was digested with HindIII and probed with the RhiXb cDNA on Southern blot (Fig 4). No obvious alteration of the restriction profiles from either D.A.A. (genotype denoted — — / — — on Fig 4, indicating two copies of the silent Rh allele) or her parents and children could be detected. Comparison of the Rh genotypes and the restriction profiles (best shown with the 7.3-kb and 6.7-kb hybridization bands) is consis-
tent with the inheritance of the silent Rh allele (---) with a chromosome carrying only the CcEe gene at the RH locus (hybridization band of 7.3 kb), as found in the RhD-negative condition (ddcece sample). In this experiment, the silent chromosome could not be distinguished from a normal RhD-negative chromosome. Obviously, a large deletion at the RH locus cannot account for this particular example of the amorphic type of Rh,,,, but it is not to be concluded that this may not occur in other individuals with a similar phenotype.

Analysis of Rh transcripts in the Rh,,,, amorph phenotype. To determine whether the lack of expression of the Rh polypeptides in the RBCs of D.A.A. might result from a mutation in the coding sequence of the CcEe gene, overlapping regions of the reticulocyte Rh cDNA of this patient and of an Rh-positive control were amplified in vitro (not shown). Amplification products of expected size obtained from the D.A.A. samples were cloned in a pUC18 vector, and several clones were sequenced to detect possible errors caused by mutations that could have occurred during amplification. No difference was seen between the nucleotide sequence of the Rh transcript from D.A.A. and the previously characterized transcript of the CcEe gene from the individual with common Rh phenotypes. Therefore, it is concluded that the absence of Rh antigens at the RBC surface of the Rh,,,, amorph phenotype is neither the result of a gene rearrangement at the RH locus nor the result of point mutations(s) in the Rh coding region.

This conclusion raised the hypothesis that the transcriptional activity of the Rh,,,, gene or the message stability may have been affected. To estimate the relative level of the Rh transcript in Rh,,,, and normal cells, Northern blot analysis was performed on RNA preparations from circulating reticulocytes of D.A.A. and the Rh-negative donor. Although the Rh mRNA species were easily detected in the control K562 and HEL human erythroleukemic cell lines, the level of hybridization of the Rh probe in reticulocyte RNA preparations was too low to ascertain whether the observed reduction of signal in the Rh,,,, sample, compared with normal reticulocytes, signified an altered Rh gene transcription (not shown). However, it is assumed that mutation(s) in regulatory elements of the Rh gene (CcEe in this patient) may have caused a drastic reduction of mRNAs encoding the Rh polypeptides. Under this hypothesis, it cannot be ruled out that the Rh,,,, silent gene could direct the synthesis of a very low amount of Rh polypeptides, and this might account for the characterization of a so called homologous Rh-like polypeptide in Rh,,,, erythrocytes recently reported by Connor et al. However, we and others have been unable to detect such a polypeptide, either by immunostaining or by radioactive surface labeling (and this work). This apparent discrepancy might result from a difference in the sensitivity of the techniques used. Further comparison of the present data cannot be done because neither the regulator nor amorph status of the Rh,,,, sample studied, nor the level of expression and the membraneous/cytosolic nature...
of the Rh-like polypeptide, were clearly discussed by Connor et al. Because Rhnull cells have a normal aminophospholipid translocase activity, but lack or are severely defective in Rh proteins, it is unlikely that Rh polypeptides and the translocase are identical molecules as proposed earlier.

Because the silent and regulator types of Rh-deficiencies occur under different genetic background, distinct mechanisms are expected to explain the very low or nonexpression of Rh proteins in each instance. However, as discussed above, the Rh membrane complex hypothesis may provide a simple explanation for the absence of the Rh polypeptides and other membrane proteins in both instances, assuming that either one of the Rh protein(s) or some as yet unidentified component(s), respectively, might be necessary for the correct transport or cell-surface expression of all these proteins as a stable complex in the membrane.

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