Rh null Disease: The Amorph Type Results From a Novel Double Mutation in RhCe Gene on D-Negative Background

By Cheng-Han Huang, Ying Chen, Marion E. Reid, and Christine Seidl

Rhnull disease, which includes the amorph and regulator types, is a rare genetic disorder characterized by stomatocytosis and chronic hemolytic anemia. We studied here a German family transmitting a putative amorph Rhnull disease gene and identified a rare mutation causing the loss-of-function phenotype. We analyzed the genomic and transcript structure of RH30, RH50, and CD47, the three loci thought to be most critical for expression of the Rh complex in the red blood cell membrane. We showed that in this family the RH50 and CD47 transcripts were normal in primary sequence. However, the RH30 locus contained an unusual double mutation in exon 7 of the RH30 gene, in addition to a deletion of the RH2 gene. The mutation targeted two adjacent codons in multiple arrangements probably via the mechanism of microgene conversion. One scheme entails a noncontiguous deletion of two nucleotides, [ATT(Thr322)→AT] and [CAC(His323)→CC], whereas the other involves a T→C transition [ATT(Thr322)→ATC] and a dinucleotide deletion [CAC(His323)→C]. They caused the same shift in open reading frame predicted to encode a shortened protein with 398 amino acids. The loss of two transmembrane domains and gain of a new C-terminal sequence are likely to alter the protein conformation and impair the Rh complex assembly. Our findings establish the molecular identity of an amorph Rhnull disease gene, showing that Rh30 and Rh50 are both essential for the functioning of the Rh structures as a multisubunit complex in the plasma membrane.

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Materials and Methods

Blood samples, phenotyping, and immunoblotting. Control blood samples were from D-positive (genotype, DCe/DCe) and D-negative (genotype, dce/dce) human blood donors. Blood samples under study were from 4 members of the Rhnull family, including 2 homozygotes (II-2, B.K., and II-3, D.R.) and 2 heterozygotes (III-1 and III-2; Fig 1). Members I-1 and I-2 are related23 but not available for molecular analysis. Retesting of the patients’ RBCs confirmed the absence of all Rh antigens. Immunoblot of RBC membrane proteins was performed as described.24 Two antibodies, LOR-15C9 for RhD25 and 2D10 for Rh50,26 were used. Peroxidase-conjugated antihuman and antianimal
Igs were used as the respective secondary antibodies for band visualization.

Nucleic acid isolation and Southern blot analysis. RNA was isolated from hemolsates and genomic DNA from leukocytes, as described.\textsuperscript{27,28} The Rh cDNA probes were as described\textsuperscript{28}; they span the 5' (exon 1-3), middle (exon 4-7), and 3' (exon 8-10 plus 3'-untranslated or UT) regions, respectively. The Rh50 and CD47 cDNA probes were as detailed previously.\textsuperscript{21} Southern blot was performed as described.\textsuperscript{28}

Reverse transcriptase-polymerase chain reaction (RT-PCR). The expression of transcripts was analyzed by RT-PCR, as described.\textsuperscript{21,29} Rh30-specific primers are shown (Table 1), and those for Rh50 and CD47 were detailed in our previous report.\textsuperscript{21} Amplification was repeated 30 cycles as follows: 94°C for 1 minute, 60°C for 30 seconds, and 72°C for 30 seconds.

Nucleotide determination and sequence analysis. Amplified cDNA and genomic products were purified by native 5% polyacrylamide gel electrophoresis (PAGE) and sequenced in both strands on a Model 373A automated sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced amino acid sequences were analyzed using the DNASIS program (Hitachi, South San Francisco, CA).

\section*{RESULTS}

\subsection*{Gross structure of Rh30 polypeptide genes.} Figure 1 shows a representative SphI blot of the Rh\textsubscript{null} family hybridized with the cDNA probe spanning the polymorphic region of Rh30.\textsuperscript{31} Notably, the homozygotes and one heterozygote each had a single 7.3-kb band retaining exons 4-7 whose intensity was comparable to that of D-negatives. This indicated that the Rh\textsubscript{null} patients lacked RhD gene but had two copies of RhCE gene (Fig 1, left). The other heterozygote inherited from her father a D\textsubscript{Ce} haplotype; thus, her D-specific (8.9 and 1.2 kb) and Ce-specific (5.4 and 1.9 kb) bands showed reduced intensity. Analysis of RH50 and CD47 showed no difference between controls and Rh\textsubscript{null} family members (gels not shown), indicating that all family members had two intact copies of Rh50 and CD47.

\begin{table}[h]
\centering
\caption{Primers Specific for Rh30 Genes}
\begin{tabular}{llll}
\hline
\textbf{Category} & \textbf{Sequence (5’-3’)*} & \textbf{Location} & \textbf{Position} \\
\hline
(I) Exon assay & & & \\
5’-UT\textsuperscript{2} & ATCGCTCCCTCAAGCCCTCAAGTA & 5’UT & 86–63 \\
Ex-1a & CTTTGTATCCTTAAAGGAGGTCATA & Ex 1 & 99-123 \\
Ex-2s & GGGCAAGATCTGAGCGTGATGGGC & Ex 2 & 151-174 \\
Ex-2a & CGGAACAGGTAGTACAGCACCCCTTC & Ex 2 & 312-335 \\
Ex-4s & attgacACAGACTACACCATGAAAT & In 3/Ex 4 & \\
In-4a & tctgagccttcgctcgccaa & In 4 & \\
In-4s & gactgtgtctccctactatct & In 4 & \\
Ex-5a & GCTGATCTCCT(C)TGTGGGATGGAC & Ex 5 & 775-798 \\
Ex-6a & ACTTATGTCAGCTGCGGGGT & Ex 6 & 802-826 \\
In-6a & tgcagttgctcGAGAGCG & In 6/Ex 6 & \\
Ex-7s & TGGTGAACCGGATGCCTGGGATT & Ex 7 & 943-966 \\
In-7a & agtgccacagccgagcagga & In 7 & \\
In-8s & tctgagaaaaacactctgtgctc & In 8 & \\
Ex-9a & CCAGAAAATGCTGCTAATCTATTT & Ex 9 & 1198-1224 \\
Ex-10s & AGTTTCTCATGTTGCTGGATT & Ex 10 & 1226-1250 \\
3’-UT\textsuperscript{D} & GTATTCTACAGTGCTATAAAATGGTG & 3’UT, D & 1432-1458 \\
3’-UT\textsuperscript{CE} & CGTCTCGACCTGTTCTTATAC & 3’UT, CE & 1363-1388 \\
II) Amplification of exon 7 & & & \\
In-6s & tttgaggtgcctagcctgttcct & In 6 & \\
Ex-7a & gttgacacatATGCCATGCGGT & In 7/Ex 7 & \\
In-7a & Same as in (I) & & \\
III) RT-PCR & & & \\
5’-UTs & CTTGGGATGAGGAGGCAGAACA & 5’UT & 33–22 \\
Ex-5a & TGGGCAAGCATCACAAGAAG & Ex 5 & 662-640 \\
Ex-4s & CAAGAATCGCAGCAGGAGCAGTA & Ex 4 & 515-539 \\
Ex-9s & GCTTGTCTCCTAATCTCAAATATGG & Ex 9 & 1153-1179 \\
3’-UT\textsuperscript{D} & Same as in (I) & & \\
3’-UT\textsuperscript{CE} & Same as in (I) & & \\
\hline
\end{tabular}
\end{table}

*Intronic sequences are denoted by lowercase letters and others by uppercase letters.
†Nucleotide positions of exon primers are accounted from the first base of initiation codon ATG.
‡Sense (s) and antisense (a) primers to 3’-UT, exon (Ex), and intron (In) are denoted.
genes. These results indicate that the amorph gene occurs on background of RHD deletion and that both copies of RHCE are defective in the Rh null patients (Fig 1, right).

To determine whether the RhD gene deletion is total or partial, we analyzed 8 of the 10 exons of Rh30 genes by PCR and diagnostic restriction cleavage (Fig 2). Whereas heterozygote III-1 had a pattern apparently identical with that of D-positives, other members lacked D-specific exons 1, 2, 4, 5, 6, 7, 9, and 10. With regard to the cleavage of exons 1 and 2, the two homozygotes contained Ce- or CE-specific exons, whereas the heterozygotes exhibited a composite pattern. Taken together, these data demonstrate a total absence of RhD gene in Rhnull patients and indicate that some subtle molecular defect(s) have silenced the phenotypic expression of their RhCE gene.

Identification of the novel mutation in RhCe gene by transcript analysis. Although being a consanguineous pedigree, the inheritance pattern of this Rhnull family (Fig 1) did not rule out the occurrence of possible mutations in related suppressor loci. Therefore, we analyzed and sequenced the Rh50 and CD47 cDNAs from the Rhnull patients. No abnormality was found in the Rh50 (Fig 3A) or CD47 transcript (not shown), conforming to the transmission of an amorph gene.

To define the nature of the amorph mutation, the Rh30 cDNA was amplified with different pairs of gene-specific primers. In both Rhnull patients, only RhCE but not RhD cDNA was detected, whose size and amount were comparable to that of controls (Fig 3A). Sequencing showed that the Rh30 cDNA was of Ce type, containing C- and e-specific polymorphisms. However, this cDNA harbored a very rare double mutation in exon 7, which targeted two adjacent codons (322 and 323) and caused a noncontiguous deletion of two nucleotides, 966T and 968A (Fig 3B). Significantly, the mutation resulted in both frameshift and premature termination. Thus, the cDNA was predicted to encode a shortened RhCe-like polypeptide of 398 amino acids (Fig 4A). The premature termination ablated the last 2 TM domains of the protein, whereas the frameshift gave rise to a new C-terminal sequence of 76 amino acids likely facing the cytoplasmic side (Fig 4B). We did not try to establish the status of the RhCe-like protein in the membrane, because no antibody can detect RhCE proteins by immunoblot. However, we showed an absence of RhD but a significant expression of Rh50 in the Rhnull patients (Fig 4C). In light of a normal structure of Rh50 and CD47, we conclude that the double deletion is the primary defect underlying the amorph Rhnull disease gene in this family.

Inheritance of amorph gene and confirmation of genomic mutation. The detection of a unique RhCe-like transcript (Fig 3) indicated that the patients are homozygous, whereas their
children are heterozygous, for the mutation. As a new BamHI site was created, we performed a diagnostic assay in the four members. Whereas no cleavage was seen in controls, a total digestion in the 2 patients and a partial digestion in their children were observed (Fig 5A). This pattern is entirely consistent with the transmission of the amorph gene in an autosomal recessive fashion. Direct sequencing of the exon 7 containing fragment showed the same noncontiguous deletion (Fig 3B), confirming homozygosity of the mutation in the 2 patients.

**DISCUSSION**

In this study, we examined a German family in which a putative amorph Rhnull disease gene is transmitted on a consanguineous background. Molecular analysis of three genetic loci relating to the expression of the Rh complex, RH30, RH50, and CD47, has led to the detection of a rare double mutation in the Rh30 gene and exclusion of possible candidate mutations in Rh50 and CD47 genes. The mutation is defined as a noncontiguous deletion of 2 nucleotides targeting 2 adjacent codons in exon 7 of the RhCe gene and thus becomes the first known amorph defect at RH30. With a deleted RhD gene positioned in cis and trans, the mutation has silenced the antigen expression of the remaining RhCe gene, causing a loss-of-function phenotype in the two Rhnull patients.

Of the double deletions identified, one hit the second or third position of codon ATT(Ile322) and the other the second position of codon CAC(His323). Based on the alignment of deletion forms, two models may be postulated to account for their origin. In spontaneous model, two hypothetical schemes could be proposed, each evoking two molecular events. In scheme I, the deletion of 966T (or 965T) and 968A is assumed to occur separately (Fig 6A). In scheme II, one event causes a transition [ATT=A TC] and the other a dinucleotide deletion [CAC=C] (Fig 6A). In the microgene conversion model, the same end product may arise by a single event in which a heteroduplex of RhD and RhCe could be formed via homologous pairing; failure in repair synthesis involving codons 322 and 323 would result in patched transfer of the RhD-specific C residue and a contiguous deletion of TC dinucleotide (Fig 6B). Although microgene conversion might be infrequent in the Rh system, it is implicated as an important mechanism for the diversity of Ig and MHC gene families and provides a plausible explanation to the origin of some rare spontaneous mutations.

The amorph mutation has targeted a nonconserved region, possibly the fifth endofacial loop, in the Rh30 polypeptides.
Thus, its result as a loss-of-function phenotype is most likely due to the inevitable shift in open reading frame. The status of the putative RhCe-like protein in the Rhnull cells remains to be established. Nevertheless, several lines of evidence suggest that the mutant protein may be inserted into the membrane but not accessible to the antibodies that are strictly conformation-dependent. (1) The RhCe-like transcript was abundantly expressed, suggesting that the steady-state level of mRNA is not affected by the mutation. (2) Albeit at a reduced level, Rh50 was readily detectable by immunoblot. As a critical interaction partner of Rh30, 12-14 this expression implies a possible association of Rh50 with the RhCe-like protein. (3) Prior surface-labeling studies detected a structurally similar Rh30 polypeptide in the Rhnull membrane, 36 indicating that the absence of Rh antigens does not necessarily mean the absence of Rh proteins. As predicted, the primary sequence of the RhCe-like protein indicates a loss of 2 TM domains and a gain of 76 new amino acids in the C-terminal region. Such structural changes would disturb the conformation of the RhCe-like protein and affect its interaction with Rh50 and other related glycoproteins in the RBC membrane.

Identification of the first amorph Rh30 gene establishes that Rh30, akin to its interacting partner Rh50, is an essential member of the Rh membrane structures. However, the molecular details regarding this protein-protein interaction remain to be elucidated. Limited proteolysis and antibody probing suggested that Rh30 and Rh50 are each composed of two 6-TM domains, forming a tetramer through N-terminal contacts. 13 However, additional contact sites are likely present to further dictate the assembly of Rh membrane structures. The amorph protein has a change in the C-terminal portion after Pro323, but its N-terminal sequence is identical with the wild-type one. Its association with the dysfunction of the Rh complex suggests a possible interaction of Rh30 with Rh50 through their C-terminal regions. Notably, the C-termini of Rh50 mutants in regulator Rhnull disease resulting from a splicing donor mutation 21 or a point
deletion are also abnormal because of the inherent frameshift and premature termination. In the former, the only difference from the Rh50 protein lies C-terminal to the 10th TM domain, whereas the latter is altered after the 11th TM domain. Missense mutations targeting the conserved C-terminal TM domains of Rh50 are also associated with regulator Rhnull (our unpublished data). These coincidental observations suggest that the C-terminal regions of Rh30 and Rh50 play a crucial role in forming the multisubunit Rh structures in the RBC membrane.

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REFERENCES

protein structure of a human blood group Rh polypeptide. Proc Natl Acad Sci USA 87:6243, 1990


20. Huang C-H, Blumenfeld OO: MNSs blood groups and major


Fig 6. Two models accounting for origin of the double mutation in the amorph Rhnull disease gene. (A) Spontaneous mutation model. Sequences of codons 318-327 in RhD and RhCe genes are shown on top. Possible arrangements of the mutated region (boxed) in the amorph gene are depicted in two hypothetical schemes (see Discussion for details). a and b denote alternatives of the same scheme. Scheme I shows a noncontiguous deletion of two nucleotides, whereas scheme II shows a contiguous deletion of 2 nucleotides in association with a T-C transition. The BamHI site is shown. (B) Microgene conversion model. A heteroduplex is formed between RhD and RhCe genes via homologous pairing and strand synapsis. A failure in repair synthesis involving codons 322 and 323 would result in A-C transition and contiguous deletion of 2 nucleotides (boxed). This model is compatible with scheme II shown above but accommodates the latter in a single event.
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