Molecular analysis of Rh polypeptides in a family with RhD-positive and RhD-negative phenotypes

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To investigate the genetic basis of the Rh polypeptide gene, we attempted the isolation of cDNA clones for Rh polypeptide from a family with the RhD-positive and RhD-negative phenotypes using the reverse transcription (RT)–PCR method for each reticulocyte RNAs followed by subcloning. The isolated cDNAs showed the existence of another Rh-related clone (RhPII-1 cDNA, tentative designation) besides the RhPI and RhPII cDNA clones reported previously by us. The RhPII-1 cDNA had a single nucleotide substitution with one amino acid substitution compared with the RhPII cDNA: substitution C→T in nucleotide 380, changing codon 127 from GCG to GTG (Ala→Val). The RhPI, RhPII, and RhPII-1 cDNA clones were detected in all

individuals by the PCR experiment. This suggests that the Rh polypeptide genes have been inherited from parents and might be highly polymorphic. The PCR amplification of an RhPII-specific region from reticulocyte RNA and genomic DNA in all the family proved that the RhPII gene exists in both RhD-positive and RhD-negative individuals. By Southern-blot analysis of the DNAs from the family, two independent polymorphisms concerning the RhC/c and RhD/d phenotypes were observed. These results demonstrate that the RhPI and RhPII genes are also present in the RhD-negative donors, and the RhPII-related cDNAs encode not the RhD, but the RhC/c and/or E/e, polypeptides.

INTRODUCTION

The Rh blood-group system is comprised of numerous inherited antigens, the most polymorphic of which are the RhD, C/c and E/e antigens [1,2]. These antigens are thiol-group-containing polypeptides that are not significantly glycosylated [3,4]. They are all carried by 30–32 kDa integral membrane proteins [3–5]. There is evidence that D, C/c and E/e are carried on three distinct homologous polypeptides [6–8], although all shared a common N-terminal sequence [7,9,10]. Also, the mRNA encoding one of Rh polypeptides was isolated and its complete primary structure was determined [11,12]. The cDNA showed an open reading frame composed of 1251 nucleotides. Hydropathy analysis of the deduced 417 amino acids sequence for Rh polypeptide indicated the existence of a high degree of hydrophobicity and predicted the presence of 12 or 13 membrane-spanning domains [11,12].

We previously reported the existence of two cDNA clones related to Rh polypeptide genes [13]. Both these two clones consisted of an open reading frame with 1251 nucleotides. One clone (designated RhPI cDNA) showed a single nucleotide substitution of A for G at nucleotide 174 without amino acid substitution as compared with the nucleotide sequence of the Rh polypeptide cDNA reported by Avent et al. and Cherif-Zahar et al. [11,12]. The other clone (designated RhPII cDNA) had 41 nucleotide substitutions following 31 amino acid substitutions. Hydropathy analysis of the deduced amino acid sequence for the RhPII polypeptide suggested the presence of 12 membrane-spanning domains.

It is as yet unclear which of these cDNAs encode which of the Rh antigens. There are several predicted extracellular amino acid sequence differences between RhPI and RhPII which could account for a novel antigen [13]. It was guessed from our previous report that the RhPI cDNA might encode the RhC/c and/or E/e polypeptides, since the RhPI gene was detected from mRNAs in the RhD-negative erythroid cells [14]. Also, the

RhPII cDNA clone probably appears to encode the RhC/c and/or RhE/e, but not the RhD antigen, because of the disagreement with a partial RhD protein sequence compared with that of the RhPII [11] and the revelation of the polymorphism associated with the RhC/c antigenic variation regardless of the RhD antigen ([13], the preceding paper [15]). In contrast, Le Van Kim et al. [16] reported that a RhXIII cDNA clone, which has a strong resemblance to the RhPII cDNA, encoded the RhD antigen. This clone had the same open reading frame as the RhPII cDNA. The RhXIII cDNA differed by 36 amino acid substitutions from the Rh polypeptide cDNA reported by Avent et al. [11] and Cherif-Zahar et al. [12], and the nucleotide positions 956–1061 showed high divergence of approx. 30 % between the two cDNAs. The nucleotide sequences of the RhPII and RhXIII cDNAs are the same in this region. Le Van Kim et al. [16] reported that this specific region was detected in the RhD-positive donors in the PCR experiment, but not the RhD-negative donors. In the present study we attempted to detect Rh polypeptides from a family with RhD-positive and RhD-negative phenotypes in order to clarify the genetic basis of the Rh polypeptide gene. We report here that the RhPII cDNA clone was detected in RhD-negative, as well as RhD-positive, donors.

MATERIALS AND METHODS

Blood samples

In the present study the pedigree of a family with the RhD-positive and RhD-negative phenotypes in the Rh blood-group system was analysed (Figure 1). The family consisted of four members of father (I.1), mother (I.2), one son (II.1) and one daughter (II.2). RBCs from II.1 and II.2 did not agglutinate with either polyclonal IgG anti-D and monoclonal IgM anti-D antibody, and proved to be negative in an indirect anti-globulin test with polyclonal IgG anti-D antibody. The Rh phenotypes of them were CcDee (CDe/cde), ccDee (cDe/cde), ccdee (cde/cde),

Abbreviation used: RT, reverse transcription.

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Figure 1 Pedigree of a family with the RhD-positive and RhD-negative phenotypes

and ccdee (cde/cde) respectively. The anticoagulated peripheral blood was obtained from the family.

PCR amplification

Total RNA was prepared from reticulocytes in peripheral blood by NH_4Cl lysis according to the method of Tse et al. [17]. Reticulocyte cDNA templates were synthesized from the RNA by reverse transcriptase and amplified by PCR using the GeneAmp RNA PCR kit (Perkin-Elmer/Cetus, Norwalk, CT, U.S.A.). Briefly, 1 μg of total RNA was incubated with 2.5 units of cloned Molony-murine-leukaemia-virus (M-MLV) reverse transcriptase and 2.5 μM oligo(dT)₁₈ for 15 min at 42 °C. The reaction mixture was then amplified by PCR (35 cycles; 1 min at 94 °C, 1 min at 55 °C, and 3 min at 72 °C) for the Rh polypeptide cDNA, then divided into two regions using two sets of Rh1-1/1-2 and Rh2-1/2-2 primers designated according to the published nucleotide sequence of Rh polypeptide cDNA [11–13]. The primers used and their positions are as follows [13]: Rh 1-1 (nts –30 to –11), 5'-AATCCCGGCCTGCACAGAGA-3'; Rh1-2 (nts 674–655), 5'-GAGTTGACACTTGGCCAGAA-3'; Rh2-1 (nts 606–625), 5'-AACGATACCCAGTTTGTCTG-3'; Rh2-2

Figure 3 Nucleotide sequence of RhPII-1 cDNA

Total reticulocyte RNA was reverse-transcribed, PCR-amplified, subcloned and sequenced by the dideoxy chain-termination method as described in the Materials and methods section. The RhPII-1 cDNA showed the substitution C→T in nt 380, changing codon 127 from GCG to GTG (Ala→Val) compared with the RhPII cDNA. An arrow shows the mutation position.

(nts 1283–1264, 5'-CAGGCCTTGTTTTTCTTGGA-3'. For the control, water was added in place of cDNA template. After amplification, 5 μl of the reaction mixture was electrophoresed in a 2% (w/v)-agarose gel. The PCR product was re-amplified using the asymmetric ratio (100:1) of the two primers for the purpose of performing the direct sequencing.

DNA sequencing

The single-stranded DNAs obtained by the asymmetric PCR amplification were sequenced by the dideoxy chain-termination method using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, OH, U.S.A.), as described

Figure 2 Direct sequencing of the PCR-amplified Rh1 and Rh2 cDNAs

To demonstrate the presence of RhPI and RhPII cDNAs in the heterozygous state, the direct sequencing of (a) Rh1 and (b) Rh2 cDNAs in child II.1 with the Rh phenotype of ccdee by way of example were shown. (a) Heterozygous positions at nts 505, 509 and 514. (b) Heterozygous positions at nts 1053, 1057, 1059, 1060 and 1061. Arrows show the heterozygous nucleotide positions.

(a) RhPII-specific region and the primers used

RhPI cDNA	976	T C C G T C A T G C A C T C C A T C T T C A G C T T G C T G
RhPII cDNA	976	T C C <u>A T C A T G G G C T A C A A</u> C T T C A G C T T G C T G
		RhPII-s
RhPI cDNA	1006	G G T C T G C T T G G A G A G A T C A <u>C</u> C T A C A T T G T G
RhPII cDNA	1006	G G T C T G C T T G G A G A G A T C A <u>T</u> C T A C A T T G T G
		RhPII-a
RhPI cDNA	1036	C T G C T G G T G C T T C A T A C T G T C T G G A A C G G C
RhPII cDNA	1036	C T G C T G G T G C T T <u>G A T A C C G T C G G A G C C</u> G G C

(b) Reticulocyte RNA

(c) Genomic DNA

(d) Nucleotide sequence

Figure 4 Amplification and sequencing of the RhPII-specific region from reticulocyte RNA and genomic DNA

(a) The region for nt 979–1062 shows a high divergence between the RhPI and RhPII cDNAs. The nucleotide sequences of sense primer RhPII-s (nts 979–992) and antisense primer RhPII-a (nts 1062–1047) are indicated in open boxes. The PCR procedure used was described in the Materials and methods section. The annealing temperature was set at 46 °C. The PCR-amplified products from (b) reticulocyte RNA and (c) genomic DNA were resolved on agarose gel: lane 1, control (CcDee); lane 2, father (I.1, CcDee); lane 3, mother (I.2, ccDee); lane 4, son (II.1, ccDee); lane 5, daughter (II.2, ccDee). (d) The RhPII-specific region amplified by the PCR method using a set of RhPII-s/RhPII-a primers were directly sequenced by the dideoxy method. An arrow shows the nucleotide position (nt 1025) of the RhPII-specific region.

by the manufacturer. In addition, an aliquot of the PCR products was purified on agarose gel and subcloned into the pCRTMII vector using the TA cloning kit (Invitrogen, San Diego, CA, U.S.A.). The plasmid DNAs of several independent clones were alkali-denatured and sequenced using M13 universal primers.

Southern-blot analysis

Genomic DNAs were extracted from peripheral-blood leucocytes of the family. DNA samples were digested with restriction endonuclease *RsaI*, separated by electrophoresis in 1.2 % agarose gel, transferred to a nylon membrane (Hybond N, Amersham International), hybridized with radiolabelled Rh1 region (nts –30 to 674) and Rh2 region (nts 606–1283) in RhPI cDNA clone as described previously and autoradiographed [15].

RESULTS

Direct sequencing of the cDNAs for Rh polypeptide from reticulocyte RNA

To investigate the genetic basis of Rh polypeptide genes using two sets of Rh1-1/1-2 and Rh2-1/2-2 primers, we attempted the

amplification of Rh polypeptide cDNAs by the PCR method from reticulocyte RNAs of a family with the RhD-positive and RhD-negative phenotypes. The PCR amplification yielded two expected PCR products composed of 704 nucleotides (Rh1-1/1-2) and 678 nucleotides (Rh2-1/2-2). No PCR-amplified product was obtained in the control containing water. The PCR-amplified products of Rh1 region (nts –30 to 674) and Rh2 region (nts 606–1283) were further re-amplified using an asymmetric ratio (100:1) of the two primers and then were directly sequenced. As the result, both the Rh1 and Rh2 cDNAs showed a very complex sequencing pattern suggestive of the presence of multiple cDNA clones, and we confirmed that all individuals were heterozygote (Figure 2). This fact indicates that the Rh polypeptide cDNAs obtained from all the family consisted of the multiple species, and the RhPI and RhPII mRNAs are present in reticulocytes regardless of the RhD-positive and RhD-negative individuals.

Subcloning and nucleotide sequencing of Rh polypeptide cDNAs

In order to separate the multiple Rh polypeptide cDNA clones, the PCR products of Rh1 and Rh2 regions obtained from reticulocyte RNAs were subcloned into the pCRTMII vector and

Figure 5 Southern-blot analysis of genomic DNA digested with *RsaI*

The digested DNAs were hybridized with the Rh1 region (nts -30 to 674) cDNA probe. The arrowhead (◄) indicates a hybridization band that is absent in the RhD-negative DNAs. An arrow indicates a hybridization band that is absent in the RhCC-phenotype DNA: lane 1, control 1 (CCDee); lane 2, control 2 (CcDee); lane 3, father (I.1, CcDee); lane 4, mother (I.2, ccDee); lane 5, son (II.1, ccdee); lane 6, daughter (II.2, ccdee). Final wash was performed at 65 °C for 15 min in 0.1 × SSPE (1 × SSPE is 0.15 M NaCl/10 mM NaH₂PO₄/1 mM EDTA) and 0.1 % SDS.

their inserts were sequenced with universal primers; 20 independent subclones for the Rh1 and Rh2 PCR products were analysed in all individuals. Consequently, the nucleotide-sequence analyses in the family revealed multiple Rh cDNA clones. Three kinds of cDNA clones to all the family were each observed in several subclones. These cDNAs were of identical size and had the open reading frame of 1251 nucleotides. The three Rh polypeptide cDNAs obtained from the family were as follows: RhPI cDNA, RhPII cDNA and a variant of RhPII cDNA (tentatively designated 'RhPII-1'). The RhPI and RhPII cDNAs encoding Rh polypeptide have already been reported by us [13]. The RhPII-1 cDNA sequence showed a single point mutation in which C changes to T at nt 380, resulting in a valine-for-alanine substitution at amino acid residue 127 as compared with the RhPII cDNA (Figure 3).

Thus the result of nucleotide-sequencing analyses for Rh polypeptide cDNAs in the family showed the presence of three cDNA clones, and proved that the Rh polypeptide genes have been inherited in the family. Also, the appearance of this Rh-related cDNA clone (RhPII-1) suggests that the Rh polypeptide genes might be highly polymorphic.

Detection of the RhPII-specific region from reticulocyte RNA and genomic DNA

To confirm that the RhPII gene is present in all the family, we attempted PCR amplification of the RhPII-specific region from reticulocyte RNA and genomic DNA (Figure 4). Between the RhPI and RhPII cDNAs, the region corresponding to nts 979–1062 showed a high divergence and, moreover, is the Rh exon-specific region [13,16]. Also, the nucleotide sequence between the RhPII and RhXIII cDNA agrees with this region. A schematic diagram for the detection of the RhPII gene is shown in Figure 4(a). The RhPII-specific primers (RhPII-s, nts 979–992; RhPII-a, nts 1062–1047) were synthesized, and a 84 bp product was amplified by PCR and analysed in agarose gel. The expected PCR product was detected in all the family, from both RNA and DNA templates (Figures 4b and 4c). The direct sequencing analysis of the PCR product showed a single nucleo-

tide substitution (C→T) at nt 1025 in comparison with the RhPI cDNA (Figure 4d). This result proved that the amplified PCR product is the RhPII cDNA. Further, the PCR products were subcloned and the inserts were sequenced. The nucleotide-sequencing analysis showed the RhPII cDNA in all of 20 independent subclones investigated (results not shown). These data demonstrate that the RhPII gene is present in both the RhD-positive and RhD-negative donors and detected in both RNA and DNA in the PCR experiment.

Southern-blot analysis

We reported in the preceding paper [15] that Southern-blot analysis using the Rh1 region cDNA probe showed polymorphisms of the RhCC-positive and RhCC-negative phenotypes in addition to those of the RhD-positive and RhD-negative phenotypes. We then performed Southern-blot analysis of the family using the Rh1 region (nts -30–674) cDNA probe (Figure 5). DNA from the family was digested with *RsaI*. As shown in Figure 5, the 2.35 kb restriction fragment was missing in the RhD-negative donors (lanes 5 and 6). In addition, the 1.9 kb restriction fragment was missing in CCDee donor (lane 1), was present in double dose in the Rhcc donors (lanes 4, 5 and 6) and in single dose in the RhCc donors (lanes 2 and 3). This result clearly indicates that the genomic polymorphism in Southern-blot analysis cannot discriminate between the RhD-positive and RhD-negative individuals. Next, Southern-blot analysis using the Rh2 region (nts 606–1283) cDNA probe showed the only polymorphism of the RhD phenotypes (results not shown). These findings appear to argue against a two-locus model for Rh inheritance.

DISCUSSION

In the present study we isolated and characterized three Rh-related cDNAs (RhPI, RhPII and RhPII-1) by subcloning and sequence analysis based on the PCR method from a family with the RhD-positive and RhD-negative phenotypes. The RhPI, RhPII and RhPII-1 cDNA clones were found in all individuals. Hydropathy analysis of the predicted RhPII-1 amino acid sequence showed that the position of amino acid substitution existed in fourth transmembrane domain (results not shown). Therefore the mutation region of this variant would appear to be unrelated to Rh antigenicity. These data indicate that the RhPI and RhPII genes have been handed down from generation to generation and that the Rh polypeptide genes might be highly polymorphic. Next, by Southern-blot analysis of the DNA from the family using the Rh1 region cDNA probe, we showed polymorphisms concerning the RhC/c phenotypes as well as the RhD/d phenotypes. These findings argue against the Rh locus being composed of two related genes [18].

Although speculative, it is presently thought that the RhPI-related cDNAs encode the C/c and/or E/e polypeptides and the RhPII-related cDNAs encode the D polypeptide [19]. However, we demonstrated in the present study that the RhPII gene transcripts were also produced by the RhD-negative individuals. Preliminary studies using the PCR-amplified reticulocyte RNAs from all individuals did not demonstrate differences in the relative amounts of the RhPI and RhPII mRNAs, implying that the stable levels of the RhPI and RhPII mRNAs are not decreased (results not shown). If the inheritance of Rh blood-group system is based on two closely linked genes and one of these two genes is missing in the RhD-negative donors, the present results argue against the above speculation. The Rh antigenic specificities of the RhPI- and RhPII-related polypeptides have not been

established. Therefore it is not clear whether the same three Rh cDNAs isolated from all the family encode which Rh antigens. The three genes (RhPI, RhPII and RhPII-1) shown in the present study are thought to encode the RhC/c or E/e polypeptide and not the RhD polypeptide, because the RhD would refer to a missing gene [15,18]. There is a possibility that the Rh-related mRNAs apart from these three mRNAs were not identified by these experiments because of their low expression in reticulocytes. We are now investigating the presence of additional Rh-related cDNA clones in all the family.

The most important fact in the present study is that the RhPII cDNA clone exists in both the RhD-positive and RhD-negative donors. A group of workers in Paris [16] recently reported a cDNA clone regarded as the RhD polypeptide. This clone (RhXIII) had an open reading frame composed of 1251 nucleotides and differed from the Rh polypeptide cDNA clone (RhIXb) they reported; it had 44 nucleotide substitutions, resulting in 36 amino acid substitutions. The RhPII cDNA clone we reported showed eight nucleotide substitutions resulting in seven amino acid substitutions as compared with the RhXIII cDNA [13]. The RhPII and RhXIII cDNA clones had very similar nucleotide sequences and topology. The Paris group characterized RhXIII cDNA by PCR amplification of nts 956–1061 that showed a high divergence. Southern hybridization with an RhXIII-specific probe (nts 1048–1062) revealed that the expected PCR product was detected in the RhD-positive donors, but not in the RhD-negative, donors [16].

We amplified the same nucleotide positions in both the RhD-positive and RhD-negative donors using the primers they designed (nts 941–955 and nts 1076–1062). The PCR products were obtained in all samples according to expectation, followed by subcloning and sequencing. Consequently, only the nucleotide sequence for the RhPI cDNA was detected, whereas that of the RhPII cDNA could not be detected at all in either the RhD-positive or RhD-negative donor (results not shown). This result strongly suggests that it is very difficult to amplify the RhPII cDNA because the primers used are the RhPI-specific primers. We therefore performed a second PCR as a template for the first PCR product, using the RhPII-specific primers (RhPII-s, nts 979–992; RhPII-a, nts 1062–1047) we designed, as shown in Figure 4(a). The PCR product essentially yielded only the 84 bp product in both the RhD-positive and RhD-negative donors, and we ascertained that its product was the RhPII gene by sequencing analysis (results not shown); the RhPII and RhXIII cDNA therefore do not encode the RhD polypeptide. Also, the protein sequences of RhPII and RhXIII deduced from their cDNAs are different (42–54 amino acid residues) as determined from sequencing the RhD polypeptide [11]. Further, the region

specific for the RhPII gene is present in the unrelated RhD-negative phenotypes of ccdEE, ccdEe, and CcdEe in the Rh blood-group system (E. Kajii, F. Umenishi and S. Ikemoto, unpublished work). All these data sustain the notion that the RhPII and RhXIII cDNAs encode the RhC/c and/or RhE/e polypeptides, but not the RhD polypeptide.

In the present study we have shown that RhPII cDNA was also present in the RhD-negative donors, and another Rh-related cDNA, in addition to the RhPI and RhPII cDNAs, was found. The discovery of this additional Rh-related cDNA strongly suggests that the Rh polypeptides show extreme polymorphism, and this fact has made the heredity of the Rh blood-group system more complex. The characterization of these multiple mRNAs and the antigenically distinct Rh phenotypes may be essential to future study of Rh polypeptides.

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