The human D antigens, one of the most clinically important blood groups, are presented by RhD protein with a putative 12 transmembrane topology. To understand the molecular basis for the complex antigenic profile of RhD protein, we expressed a series of RhD fusion proteins using different portions of Duffy protein as a tag in erythroleukemic K562 cells. Because the reactivity of monoclonal anti-RhD antibody, LOR15C9, depends mainly on the sequence coded by exon 7 of RhD, we altered DNA sequence corresponding to the amino acid residues 323–331(A) and 350–354(B) in the exon 7. The mutation in region B resulted in a severe reduction in LOR15C9 binding by flow cytometry analysis, suggesting that region B may play an important role in constituting antigen epitopes recognized by LOR15C9. On the other hand, a slight decrease in the antibody binding was observed for the region A mutant, suggesting that the intracellularly located region A may elicit a long distance effect on the formation of exofacial antigen epitopes. In addition, using various monoclonal antibodies against RhD, we compared the antigenic profile of expressed RhD fusion protein with that of endogenous RhD in K562 cells as well as in erythrocytes.

As one of the most important blood group systems involved in blood transfusion and hemolytic disease, the Rh antigens are present on the erythrocyte surface as a complex including Rh30 proteins (RhD and RhCE) and Rh50 glycoprotein (see recent reviews in Refs. 1 and 2). Hartel-Schenk and Agre (3) first demonstrated that the Rh polypeptides migrated through sucrose gradients as a complex with an apparent $M_r$ of 170,000. Based on proteolytic digestion and immunoblotting, Eyers et al. (4) proposed a model that consists of two of each Rh30 and Rh50 molecules, with their N-terminal portions forming the core of the complex. The genes encoding RhD and RhCE are located at chromosome 1p34-p36 (5), whereas RH50 gene is at chromosome 6p11–21 (6). These three genes share not only a similar exon-intron composition but also homology in their deduced amino acid sequences (92% identity between RhD and RhCE, and 36% identity between Rh30 and Rh50) (2). Therefore, it appears that all three of these molecules belong to a family of structurally related membrane proteins.

The multiprotein complex provides the basis for enormously complicated D antigenic epitopes, which have been serologically classified into nine epitopes, epD1 to epD9 (7). Most of the information regarding the molecular basis for D epitopes has been derived from genetic analysis of RhD variants where part of the RhD gene is missing or replaced by the highly homologous RhCE sequence (2). Thus, D$^{VI}$ phenotype, characterized as lack of epD1, 2, 5, 6, 7, and 8, contains rearranged RHD gene where its exons 4–6 are replaced with RHCE equivalents (8). In D$^{IVa}$ phenotype that lacks epD 1, 2, 3, and 9, there is a rearrangement of exon 3 and part of exon 7 of the RHD gene (9). These observations suggest that serologically defined D epitopes are organized into overlapping motifs. A similar notion was deduced from the analysis of phage-displayed anti-RhD antibodies (10).

With the expression of Rh30 on the surface of K562 cells (11), it is now possible to elucidate the molecular basis for the D epitopes using the approach of molecular biology. However, it has been reported (12) that K562 cells express endogenous RhD protein, as detected by reverse transcription-PCR and surface binding of anti-RhD. On the other hand, the attempts to express RhD on the surface of nonerythroid cells, which do not produce endogenous RhD protein, have met little success (13, 14). It is possible that multiple proteins are required during RhD synthesis for the formation of the RhD complex on the cell surface recognized by specific antibodies. Therefore, to express a high level of RhD protein, which is biochemically distinguishable from its endogenous counterpart in K562 cells and useful in the study of the molecular basis for D epitopes, we expressed RhD as a fusion protein attaching part of Duffy protein (Fy) to the N terminus of RhD. Here, we present the data about the surface expression of RhD fusion proteins in K562 cells and the comparison of its antigenic profile with that of its endogenous counterpart in K562 cells and mature erythrocytes. Furthermore, this expression system was used in determining the amino acid residues involved in the binding of the monoclonal anti-RhD, LOR15C9 (15).

**MATERIALS AND METHODS**

**Construction of Expression Plasmids for RhD Fusion Proteins**—The coding sequence for RhD was subcloned from pcDNA3-RhD into pREP4 vector at KpnI and XbaI restriction sites, generating the plasmid pR-RhD. Similarly, the plasmid pR-Fy was created by subcloning the Duffy (Fy) coding region from pcDNA1-Fy into pREP4 at HindIII and NotI. The plasmid pR-DD1 coding for the full-length Fy and RhD proteins tandemly linked (Fig. 1) was constructed as follows. Using pR-RhD as a template and P-1 (see Table I) and REP-R (reverse primer located downstream from the insert in the vector) as primers, the first PCR reaction was carried out according to previously described conditions (16). Using the first PCR product as a mega-primer, plus template pR-Fy and primer REP-F (a forward primer located upstream from the insert) the second PCR reaction was performed, generating a DNA
fragment designated as DD1-PCR product (Fig. 2). As detailed in the Fig. 2 legend, the plasmid pR-DD1 was constructed via a three-fragment ligation at three restriction sites, AseI, BbsI, and BglII. The sequence between BbsI and BglII in pR-DD1 was verified by an automated DNA sequencing. By applying a similar strategy, the plasmids pR-DD2 and pR-DD3, which code for the fusion proteins DD2 and DD3, respectively (Fig. 1) were constructed. In the construction of these two plasmids, the primers P-2 and P-3 (see Table I) were used in the two-step PCR amplification, and the restriction sites HindIII, rather than BbsI, was used in the three-fragment ligation.

**Substitution of Specific Regions in the RhD Exon 7 with Corresponding Sequence from RhCE**—Two plasmids, pR-DD2A and pR-DD2B expressing DD2 proteins mutated in regions A and B of RhD exon 7, respectively, were constructed. The plasmid pR-DD2 was used as a template in two PCR reactions with two sets of primers (see Table I): P-A and REP-R; P-Ac and REP-F. The two fragments thus generated were then linked by PCR because of their 17-nucleotide overlapped region (underlined sequence in P-A and P-Ac in Table I). The final PCR product was digested with BglII and NotI and subcloned into pR-DD2, generating the plasmid pR-DD2A. pR-DD2A has the same sequence as pR-DD2 except for the region A in RhD exon 7 (Fig. 3), as confirmed by double strand DNA sequencing. A second plasmid, pR-DD2B, coding for DD2 mutated in region B of RhD exon 7 (Fig. 3), was constructed by using primers P-B and P-Bc (see Table I) according to the same procedure described above.

**Cell Culture and Transfection**—K562 erythroleukemic cells were transfected with 1 μg of plasmid and 10 μl of LipofectAMINE Reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. After incubating the cells for 48 h at 37 °C and 5% CO₂ in RPMI medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), stably transfected cells were selected by culturing diluted cells (1:10) in the presence of 200 μg/ml of hygromycin (Boehringer Mannheim) for 2 weeks. The plasmids pREP4 (“mock”), pR-RhD, pR-Fy, pR-DD1, pR-DD2, and pR-DD3 were used in the K562 cell transfections.

**Flow Cytometry Analysis of Transfected Cells**—K562 cells (5 × 10⁶) suspended in 50 mM phosphate buffer with 0.5% saline (PBS) containing 0.5% bovine serum albumin and 0.01% sodium azide were incubated with either human monoclonal antibody (LOR15C9, 3 μg/ml) or a mouse monoclonal antibody recognizing Fy6 (anti-Fy6, 9 μg/ml) at
**RhD Fusion Protein**

The numbers directly below the DNA codon represent the position of corresponding amino acid residues. The arrows above the sequences indicate the junction between Fy (5' region) and RhD (3' region). Note that the P-3 includes an extra restriction site, SpeI, between the Fy and RhD regions. P-A and P-B were designed based on the coding sequence of RhCE, whereas P-Ac and P-Bc were derived from the noncoding sequence. P-A and P-Ac are complementary to each other in the region underlined. The same is true with P-B and P-Bc. The nucleotides that are different from RhD are in bold type.

### Table I

**Oligonucleotides used in the construction of plasmids**

| Oligonucleotides used in construction of plasmids for RhD fusion proteins | 5'-ACC CTT GGA AGC AAA TCC ↓AGC TCT AAG TAC CCG CGG-3' |
|---|---|
| P-1 | 333 |
| P-2 | 338 |
| P-3 | 7 |

| Oligonucleotides used in construction of RhD mutants |
|---|
| P-A | 5'-C ATC TCC GTA ATG CAC TCC ATC TTC AGC TTG-3' |
| P-Ac | 333 |
| P-B | 5'-T CAT ACT GTA TGC AAC GGC AAT CCC CAG-3' |
| P-Bc | 349 |

37 °C for 30 min. After washing the cells three times in the same buffer, the cells were incubated with the appropriate secondary antibody, either sheep anti-mouse IgG conjugated to fluorescein isothiocyanate (Sigma) or goat anti-human IgG (F(ab')2), conjugate to phycoerythrin (Biosource) for 30 min at room temperature. The cells were then washed and analyzed by flow cytometry (Becton Dickinson). The control cells used in the study are K562 cells transfected with the vector, pREP4.

Confocal Microscopic Analysis of Transfected K562 Cells—Cells were fixed in a 1% paraformaldehyde solution for 20 min on ice. After washing with PBS buffer, the cells were incubated in PBS containing 0.1% saponin for 30 min at room temperature. The cells were then washed with the same PBS prior to antibody sensitization as described under “Flow Cytometry Analysis of Transfected Cells.” To prepare slides, 1 × 10⁵ cells were cytospun onto poly-L-lysine coated slides, mounted with tachrome film.

Immunoprecipitation of ⁵⁸S-Labeled Cells—Stable transfected cells (1 × 10⁵) were labeled for 2 h with 200 μCi/ml of [⁵⁸S]methionine (NEN Life Science Products), washed three times with PBS, and lysed using 20 mM Tris-HCl buffer, pH 7.4, containing 1% Triton X-100, 0.25% SDS, 1 mM tosylphenylalanyl chloromethyl ketone, 1 mM phenylmethylsulfonyl fluoride, and 100 units/ml Trasylol. After preclearing the supernatant using normal rabbit serum and protein A-Sepharose, the lysate was incubated overnight at 4 °C with either rabbit anti-Rh polyclonal antiserum or normal rabbit serum. The immune complexes were isolated by subsequent incubation with protein A-Sepharose, eluted, and separated by 12% SDS-PAGE as described previously (14).

### RESULTS

Flow Cytometric Analysis of K562 Cells Expressing RhD Fusion Proteins—To study the expression of RhD protein in K562 erythroleukemic cells and distinguish recombinant from endogenous RhD, we constructed expression plasmids for RhD fusion proteins (DD1, DD2, and DD3). As shown schematically in Fig. 1, the full-length RhD of 417 amino acid residues is linked at its N terminus either to the full-length (338 amino acids) or to the N-terminal portion (97 amino acids) of Fy, generating the fusion proteins, DD1 and DD2, respectively. Further truncated from DD2, DD3 is composed of the amino acid residues 1–64 from Fy and 32–417 from RhD. The DNAs coding for these fusion proteins were subcloned into the vector pREP4 generating pR-DD1, pR-DD2, and pR-DD3 plasmids, as detailed under “Materials and Methods.” The pREP4 was chosen as the expression vector for its strong Rous sarcoma virus promoter and its multi-copy extra-chromosomal replication ability. Table I lists the oligonucleotides used in the construction of these plasmids. After stable transfected cell lines were established, the presence of specific plasmid in the transfected cells was confirmed by PCR (data not shown). Approximately 50 cells were used as a template in the PCR with a specific Rh primer and a primer corresponding to the Rous sarcoma virus promoter region to ensure that the amplified signal was from the plasmid rather than an endogenous sequence.

To detect the expressed RhD on the K562 cell surface and distinguish it from the endogenous protein in flow cytometry, we varied a number of reaction parameters, including the amount of primary antibody, binding temperature and time, washing conditions, and secondary antibodies. Although incubation of anti-RhD antibody, LOR15C9, at higher concentrations resulted in higher mean fluorescence intensity (MFI), the MFI ratio for the expressed RhD antigen versus its endogenous counterpart diminished. Thus at 3 μg/ml of purified LOR15C9, the MFI ratio was 3.18, whereas the ratio dropped to 1.28 when the antibody concentration increased to 27 μg/ml. In addition, three different fluorescence (fluorescein isothiocyanate, Texas Red, and phycoerythrin) conjugated to secondary antibodies were tested. Under the same conditions, the best result was obtained by using phycoerythrin-conjugated secondary antibody, where the MFI is at least six times higher than using fluorescein isothiocyanate conjugate antibody. Following the conditions detailed under “Materials and Methods,” we obtained a profound shift in the fluorescence-activated cell sorter profile of expressed RhD protein over its endogenous counterpart on the K562 cell surface.

As shown in the first graph of Fig. 4A, the K562 cells transfected with the vector pREP4 demonstrated stronger binding with LOR15C9 (peak e) than with control IgG (peak c) at the same concentration. This corresponds to an increase in MFI value from 7.44 to 26.29 (Table II), indicating the presence of endogenous RhD on the K562 cell surface. Therefore, serving as a base line, peak e was added to each graph in Fig. 4A. Although no endogenous Fy antigen was detectable under the applied conditions (the first graph in Fig. 4B), the same reasoning and procedure were applied in constructing Fig. 4B. Cells transfected with either pR-RhD or pR-Fy demonstrated significant shifts of their fluorescence-activated cell sorter profile when treated with LOR15C9 or anti-Fy6, respectively, suggesting the expression of both antigens on the transfected cell surface. Among the three fusion protein-expressing cell lines, the surface expression of DD2 and DD3 was positively identified by using both LOR15C9 and anti-Fy6. For the pR-DD2 transfected cells, the binding of LOR15C9 resulted in an increase of MFI from 26.29 to 70.02, whereas the binding of anti-Fy6 caused an increase from 9.39 to 32.25 (Table II). On the other hand, the surface expression of DD1 was not detected using LOR15C9, although a slight increase in MFI (from 9.39 to 14.17) was observed by the binding of anti-Fy6.

Confocal Microscope Analysis of Transfected Cells—As
shown in Fig. 5, the presence of endogenous RhD antigen in K562 cells was demonstrated by comparing panels 1 and 2 (control IgG and LOR15C9 as primary antibody, respectively). Consistent with flow cytometric analysis, Fy antigen was not detected (panel 3) in K562 cells using anti-Fy6. Panels 4–7 in Fig. 5 depict the fluorescence of DD2- and DD3-expressing cells treated with either LOR15C9 or anti-Fy6. Both cell lines demonstrated strong binding with either antibody, and the labeling seems more predominant on the edge of the cells. The data provide further evidence that RhD fusion proteins, DD2 and DD3, were expressed on the surface of transfected K562 cells.

**Flow Cytometry Analysis of Stably Transfected K562 Cells.** Cells expressing mock, RhD, Fy, DD1, DD2, and DD3 were incubated with either LOR15C9 (A) or anti-Fy6 (B) and analyzed by flow cytometry. The results are shown as the fluorescence intensity (y axis) plotted against the counts of events (x axis). The name of each cell line is displayed at upper right corner of each graph. The peak marked c in the first graph of both panels is a control by using normal IgG as a primary antibody. In the same graph, the peak marked e was generated by using LOR15C9 (A) or anti-Fy6 (B) and thus displayed in all graphs as a reference.

**Immunoprecipitation of the Transfected K562 Cells.** Two experiments were carried out to provide biochemical evidence for the expression of RhD fusion proteins in transfected cells. Cells were labeled with [35S]methionine and immunoprecipitated with a rabbit polyclonal anti-Rh antibody. The immunoprecipitates were then subjected to SDS-PAGE followed by autoradiography as shown in Fig. 6. The immunoprecipitate of cells transfected with pR-RhD (lane 1) contained a band (marked a), which migrated as RhD protein (32 kDa). A band of 70 kDa (marked b) was detected from pR-DD1 transfected cells (lane 2), whereas a band (marked c) in the range of 50–55 kDa was visualized from cells expressing DD2 and DD3 (lanes 3 and 4, respectively). The apparent molecular masses of these bands are in agreement with estimated molecular masses of RhD fusion proteins, DD1, DD2, and DD3. All of the immunoprecipitates contained high molecular mass proteins as visualized on the gel. Although the nature of these proteins have yet to be identified, they may represent aggregates of RhD or the fusion proteins or other components involved in the formation of an RhD antigen complex in the cell membrane.

A second approach involved a preparation of membrane fractions from transfected cells following the procedure of Chaudhuri et al. (18). This fraction was resuspended in the lysis buffer for immunoprecipitation with rabbit polyclonal anti-Rh, followed by Western blotting using anti-Fy6. As shown in Fig. 7, the samples from DD1-expressing cells (lane 3) and DD2-expressing cells (lane 4) contained distinctive bands marked b and c, respectively, which are consistent with the estimated molecular masses of DD1 and DD2 proteins. The high molecular mass smear visible in lanes 3 and 4 may represent the aggregates involving Rh fusion proteins. On the other hand, no visible bands were detected from cells transfected with pREP4 (lane 2) following the same procedure. This is expected because there is no protein in the cells that can be recognized by both antibodies. Red cell ghosts (10 μl) were directly loaded in lane 1 as a control for the ECL Western blot using anti-Fy6.

**Antigenic Profile of RhD on the Surface of K562 Cells.** Although the surface expression of RhD was confirmed by the binding of LOR15C9, the extent to which the RhD on K562 cells resembles its counterpart on red blood cells had yet to be established. Toward that end, we were able to address this issue by flow cytometry analysis using a series of monoclonal antibodies against different epitopes of RhD (19) (Table III). The reactivity of each antibody was first verified by using red blood cells (RhD+) under our assay conditions. By comparing MFI values of K562 with background (without primary antibody), we then showed that seven antibodies reacted positively, whereas the remaining two (LOR11-12E2 and LOR17-6C7) failed to bind to K562 cells. Furthermore, when DD2-expressing cells were examined, the same antibody binding pattern was observed. Although the two “negative” monoclonal reagents remained negative, the rest showed a more than 3.5-fold increase compared with K562 cells. Thus, the discrepancy in the RhD antigenic profile between red cells and K562 cells is clearly demonstrated.

**Substitution of Specific Regions in RhD Exon 7 with Corresponding Sequence from RhCE.** Because LOR15C9 specifically recognizes RhD exon 7 but not the highly homologous RhCE, it is reasonable to assume that antibody-binding epitope(s) in RhD exon 7 reside in the regions that differ between these two Rh proteins as shown in Fig. 3. The alignment of exon 7 sequences of RhD and RhCE illustrates two major clusters of sequence variation, designated as regions A and B, together with two other single amino acid alterations at positions 314 and 342. Using synthetic oligomers and overlapping
PCR, we generated a mutated DD2 molecule named DD2A, where RhD sequence in region A was replaced with corresponding RhCE sequence. Similarly, we constituted DD2B, where region B was replaced with RhCE.

After confirming the surface expression of DD2A and DD2B on stably transfected K562 cells by using anti-Fy6, we examined the possible effect of mutations in RhD exon 7 on the binding of LOR15C9 in flow cytometry analysis. As suggested in Fig. 8, the antibody binding was weaker to the DD2A-expressing cells (4) than to the DD2-expressing cells (3). More strikingly, mutation in region B (5) resulted in severe reduction in antibody binding.

**DISCUSSION**

Our objective was to design a RhD fusion protein that can be detected with antibodies against both RhD and the fused tag in flow cytometry analysis and be easily distinguishable in size from endogenous RhD on SDS-PAGE. Because the RhD molecule contains 12-transmembrane domains with both termini facing cytoplasm, the fusion of a simple tag such as hexahistidine at either end would not provide a marker detectable on the cell surface. Therefore, we explored the possibility of using a type I transmembrane protein in the construction of RhD fusion proteins. Fy protein, a blood group antigen and a promiscuous chemokine receptor, has been expressed on the surface of K562 cells that does not have detectable background (18, 20). The extracellularly located N-terminal domain (64 residues) of the protein is recognized by anti-Fy6. Furthermore, the antibody binding site has been mapped to the region between

| RhD Fusion Protein | Percentage | MFI | Percentage | MFI |
|--------------------|------------|-----|------------|-----|
| Control            | 25.03 (0.77) | 26.29 (7.44) | 0.64 | 9.39 |
| RhD                | 92.77 | 143.61 | 0.32 | 6.25 |
| Fy                 | 35.18 | 25.88 | 37.42 | 72.68 |
| DD1                | 44.22 | 33.09 | 4.44 | 14.17 |
| DD2                | 83.97 | 70.02 | 36.95 | 32.25 |
| DD3                | 74.36 | 58.11 | 25.27 | 28.67 |

**FIG. 5.** Confocal microscope analysis of transfected K562 cell lines. All cell samples were fixed and then treated with saponin and subjected to immunofluorescence analysis as described under “Materials and Methods.” Mock and DD2- and DD3-expressing cells were labeled with LOR15C9 (panels 2, 4, and 6, respectively) or with anti-Fy6 (panels 3, 5, and 7, respectively). Mock cells were labeled with normal IgG as a background control (panel 1).

**FIG. 6.** Detection of RhD fusion proteins in transfected K562 cell lysates. Cell lysates were prepared from [35S]methionine-labeled cells and immunoprecipitated with rabbit anti-Rh antiserum as described under “Materials and Methods.” Lanes 1–4 depict SDS-PAGE analysis of immunoprecipitates isolated from cells expressing RhD, DD1, DD2, and DD3 cells, respectively. Molecular mass standard (M) are displayed in kDa on the left of the figure.

**FIG. 7.** Detection of expressed fusion proteins using two antibodies. The plasma membrane fractions were prepared from $5 \times 10^7$ culture cells, and the immunoprecipitation was carried out by using rabbit anti-RhD antibody as described under “Materials and Methods.” The eluates from the Sepharose beads were subjected to SDS-PAGE for ECL Western blotting with anti-Fy6. Lane 1 is red cell ghosts control for immunoblotting with anti-Fy6. Lanes 2–4 are immunoprecipitates from mock and DD1- and DD2-expressing cells, respectively. The molecular mass standards in kDa are listed on the left of the figure.

**TABLE II**

Mean fluorescence intensity and relative fluorescence derived from the flow cytometry analysis shown in Fig. 5

| RhD Fusion Protein | Percentage | MFI | Anti-Fy6 | Percentage | MFI |
|--------------------|------------|-----|----------|------------|-----|
| Control            | 25.03 (0.77) | 26.29 (7.44) | 0.64 | 9.39 |
| RhD                | 92.77 | 143.61 | 0.32 | 6.25 |
| Fy                 | 35.18 | 25.88 | 37.42 | 72.68 |
| DD1                | 44.22 | 33.09 | 4.44 | 14.17 |
| DD2                | 83.97 | 70.02 | 36.95 | 32.25 |
| DD3                | 74.36 | 58.11 | 25.27 | 28.67 |
transferred with the vector pREP4 were incubated without (plotted as fluorescent intensity against counts of events. The K562 cells human IgG (H and L chains) conjugated to phycoerythrin.

The data derived from flow cytometric and confocal microscopy analyses indicate that fusion proteins DD2 and DD3 are recognized by LOR15C9 and anti-Fy6. This underscored the notion that these two fusion proteins have the membrane topology as projected in Fig. 1. The expression of these two fusion proteins was further confirmed by the immunoprecipitation experiments. As shown in Fig. 6, both proteins migrated to approximately the same position on SDS-PAGE, although the calculated molecular mass of DD2 is approximately 7 kDa more than that of DD3. This may be due to the fact that Rh protein, which constituted mostly of DD2 and DD3, is known to migrate in SDS gel anomalously (23). On the other hand, although the expression of DD1 (80 kDa) was confirmed as shown in Figs. 6 and 7, its presence on the cell surface was not definitely identified by flow cytometric analysis. Because DD1 is composed of 19 transmembrane domains (Fig. 1) based on the model for Fy and RhD proteins, the fusion protein may be simply too big to be transported to the cell surface.

As an important molecule in the Rh complex, Rh50 is endogenously produced in K562 cell line and migrates as a protein of 32 kDa on SDS-PAGE presumably due to incomplete glycosylation. However, the extent to which the Rh50 glycoprotein is required for the expression of RhD and D epitope formation on the surface of K562 cells has yet to be elucidated. In our immunoprecipitation experiment (Fig. 6, lanes 2–4), although no band around 32 kDa is visible, the possibility that Rh50 was coprecipitated with RhD fusion proteins could not be excluded. Compared with overexpressed fusion proteins, endogenous Rh50, as well as endogenous RhD, could be simply too low in quantity to be detected under experiment conditions. Nevertheless, RhD fusion proteins with different molecular masses may prove useful in the study of interactions between Rh50 and RhD.

Smythe et al. (11) reported the expression of RhD and RhcE gene products by retroviral transduction of K562 cells. Their data provide the first direct evidence that the putative RhD gene gives rise to D and G antigens and that the putative RhcE gene gives rise to c and E antigens. They concluded that retroviral delivery of the gene was of critical importance in achieving expression of Rh antigens on K562 cells. Based on their conditions, anti-RhD bound more strongly to the RhD-expressing K562 cells than to the control cells, with an increase in MFI from 3.3 to 15.2, whereas the level of endogenous Rh antigen in the untransduced K562 cells was minuscule (from 2.8 to 3.8). On the other hand, based on our data, we were able to clearly demonstrate not only endogenous RhD in K562 cells (increase in MFI from 7.44 to 26.29) but also exogenous RhD expressed on the cell surface (increase in MFI from 26.29 to 143.61). Our data suggest that successful expression and detection of RhD protein on the K562 cell surface may have resulted from optimized fluorescence-activated cell sorter conditions with a
highly specific and purified antibody, as well as an efficient transfection system.

Because the DD2 molecule contains the full-length RhD and the junction between RhD and the Fy tag is intracellularly located, DD2 is more likely than DD3 to retain the native D antigenic profile. In addition, although RhD was also expressed under the same conditions, we believed that the DD2 molecule would be preferable in the study of the molecular basis for RhD antigenic epitopes. This is because the Fy portion at the N terminus of DD2 can serve as a tag for confirming the cell surface expression, regardless of any changes made in the RhD portion of the fusion protein. Our mutagenesis study indicated that the binding of LOR15C9 was adversely affected by both mutations, DD2A and DD2B, with the latter being more severe.

It is interesting to note that according to the proposed membrane topology of RhD protein, region B is located at the sixth extracellular loop of RhD, whereas region A is near the intracellular end of a transmembrane domain. Thus, our data suggest that region B may be directly involved in the binding with LOR15C9, whereas mutations in region A may elicit a long distance effect on antibody binding on the cell surface. In other words, the substitution of region A in RhD with corresponding RhCE sequence may transform an exofacial D epitope into “CE-like” conformation. Monoclonal antibody LOR15C9 has been well characterized by Apoil et al. (15). Their data indicate that the antibody recognizes not only RhD molecule on the cell surface in flow cytometry analysis but also the denatured proteins by immunoblotting.

Our observations that both mutations (A and B) decreased but did not abolish the binding of LOR15C9 as determined by flow cytometry further support the notion that the binding of LOR15C9 depends on conformation as well as sequence of the antigen within RhD protein.

Our analysis of the RhD antigenic profile of K562 cells suggests subtle changes in the conformation or microenvironment of RhD (both endogenous and exogenous) on the K562 cell surface in comparison with its counterpart on red blood cells. Specifically, the epD2 and 4 recognized by LOR11-12E2 and LOR17-6C7, respectively, may have been somehow affected by K562 cells. The lack of activity for LOR12E2 was further confirmed by a related antibody, LOR-12E2-1C6 (data not shown). Although Mag 1–123 may possibly have an overlapping epD specificity with LOR11-12E2, there is evidence suggesting that these two reagents are serologically distinctive (24). In addition, we tested four different monoclonal reagents with the same specificity (epD6 and 7). As expected, they all reacted positively with expressed DD2, as well as with endogenous RhD on the K562 cell surface. The information generated from such antigenic profile analysis will be important in designing approaches and interpreting data to determine the molecular basis for RhD antigenic epitopes using K562 cells as an expression host.

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