δ Glycophorin (Glycophorin B) Gene Deletion in Two Individuals Homozygous for the S–s–U– Blood Group Phenotype

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Blood cells from two unrelated individuals whose erythrocytes exhibit respectively N S–s–U– and MN S–s–U– blood group phenotypes were examined by immunoblotting, periodic acid-Schiff (PAS) staining, and Southern blotting. Protein bands characteristic of δ glycophorin (glycophorin B) were absent from the immunoblots of whole erythrocyte lysates when probed with polyclonal glycophorin antisera and from isolated erythrocyte membranes stained with PAS reagents. Genomic DNA from the two individuals’ leukocytes was digested with a panel of restriction enzymes and probed with α M glycophorin cDNA obtained from human K562 leukemic cell line. The EcoRI, PstI, and KpnI restriction site patterns were identical to those of S–s–U– controls in fragment numbers and relative size but differed from controls in band intensities. Restriction mapping with HindIII, PvuII, SacI, MpiI, and BamHI revealed that S–s–U– individuals lack fragments that are reproducibly observed in S–s–U– controls, and most likely encode δ glycophorin. Using truncated S’ and S glycophorin cDNA segments as probes and comparing, in control individuals, hybridization intensities of fragments with amino acid sequence homologies, we have inferred the assignment of restriction fragments to the α and δ glycophorin genes. Our results suggest that the absence of δ glycophorin in the two S–s–U– individuals is a result of deletion of the entire δ glycophorin gene. This is the first report of a glycophorin gene deletion.

MATERIALS AND METHODS

Blood samples. Blood samples from two unrelated S–s–U– donors (M.E.K. and M.L.A.) and certain controls (S–s–U– plus) were provided by the Community Blood Center, Dayton, OH. Control blood samples were also obtained from laboratory personnel. The MN S blood group phenotypes of the variant erythrocytes were: donor M.E.K., M–N–S–s–U–; donor M.L.A., M+N+S–s–U–. Serologic typing was performed using sera available at the Consultation Laboratory of Gamma Biologicals. M and N status was tested with antisera from human, animal, and lectin sources. Anti-S, anti-s, and anti-U were human source reagents, and at least three samples of each were used. U-negative status was confirmed by absorption-elution studies.

Polycrylamide gel electrophoresis (PAGE) and immunoblot analyses. Fresh erythrocytes were washed three times with phosphate-buffered saline (PBS) containing inhibitors of proteolysis and lysed by mixing 0.025 mL packed erythrocytes with 0.75 mL 1 x (sodium dodecyl) sulfate (SDS)-polycrylamide gel) sample buffer.20 Ten microliters of the whole lysate was subjected to electrophoresis on SDS-10% polycrylamide gels.21 Gels were blotted onto nitrocellulose as described by Towbin et al.22 and glycophorins were visualized by autoradiography after consecutive incubations with one of two polyclonal rabbit antisera and then with 125I-labeled protein A.24 One antiserum was raised against intact MN glycophorin, the second against the carboxyl terminal peptide of MN glycophorin (peptide C, residues 82 through 131).26 Whereas the glycophorin antiserum reacts with both α and δ glycophorins, the antipeptide C serum detects only α glycophorin and shows no crossreaction with δS or δ glycophorin.20,21

Erythrocyte membranes were prepared as previously described.4 To 0.50 mL membrane suspension was added 0.25 mL 3 x sample buffer and 10 μL subjected to electrophoresis. Gels were stained with Coomassie blue or periodic acid-Schiff (PAS) reagent.

Genomic DNA restriction mapping analysis. Genomic DNA was isolated from the peripheral leukocytes of EDTA-anticoagulated blood as described by Kunkel et al with minor modifications. All DNAs were larger than 50 kilobases (kb) using intact X DNA as marker.

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five residues of a glycophorin, which contains a total of 131 residues. A partial restriction map of glycophorin cDNA inserts. Restriction endonucleases are denoted as P, PstI; M, Mbol; R, RsaI; H, HpaI; and S, SacI. (B) Probes used for nucleic acid hybridization. Roman numerals designate probes. Probes I through IV were generated by PstI; probes V through VII were generated as described. Numbers at beginning and end of each probe indicate amino acid residues of glycophorin they encompass. cDNAs lack the codons of the last five residues of α glycophorin, which contains a total of 131 residues.

Restriction endonucleases are denoted as P, PstI; M, Mbol; R, RsaI; H, HpaI; and S, SacI. (B) Probes used for nucleic acid hybridization. Roman numerals designate probes. Probes I through II were generated by PstI from glycophorin cDNAs, pHGpA-1, and pHGpA-3, respectively; probes III through IV were generated by PstI, Rsal, and SacI. Numbers at beginning and end of each probe indicate amino acid residues of glycophorin they encompass. cDNAs lack the codons of the last five residues of α glycophorin, which contains a total of 131 residues.

Genomic DNAs were digested with restriction endonucleases (New England BioLab, Beverly, MA; Boehringer Mannheim, Indianapolis), under conditions recommended by the manufacturers, in a ratio of 4 to 5 U enzyme/μg DNA at 37°C for 15 hours. Blotting transfer of DNA fractionated by agarose gel electrophoresis was performed as previously described.

Recombinant plasmids, pHGpA-1, and pHGpA-3 harboring the glycophorin cDNAs, were isolated from transformed Escherichia coli RRI cells and further purified by cesium chloride density-gradient centrifugation as described. A partial restriction map of α M glycophorin cDNA inserts was predicted from their nucleotide sequences (Fig 1A). The cDNA hybridization probes are depicted in Fig 1B (I through V). The probes were removed from the plasmids by appropriate restriction enzymes, purified by electrophoresis in low-melting agarose (Bio-Rad, Richmond, CA) or 6% to 8% polyacrylamide gels, excised and labeled with [α-32P]dCTP (Amersham, Arlington Heights, IL) using random primers (Pharmacia, Piscataway, NJ) and Klenow fragment of DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, MD) as described by Feinberg and Vogelstein. The specific activities of these primer-extended probes ranged from 2.1 × 106 to 3.4 × 107 cpm/μg.

Nucleic acid hybridizations were performed essentially as described. The hybridization and washing techniques were standardized as follows: To each blotted nitrocellulose filter (~200 cm2) was added 10 mL hybridization solution containing 5 × SSC (1 × SSC = 0.15 mol/L NaCl + 0.015 mol/L Na3 Citrate, pH 7.0), 5 × Denhardt (1 × Denhardt = 0.1% Ficoll, 0.1% polyvinylpyrrolidone (PVP) and 0.1% bovine serum albumin, BSA), 10 mmol/L EDTA (pH 8.0) and 200 μg/mL sonicated salmon sperm DNA. Prehybridization was carried out at 65°C for ~10 hours; hybridization was carried out overnight at the same temperature, following addition to the hybridization solution of the appropriate radioactive probe at a final concentration of 4 to 5 × 106 cpm/ml. Highly stringent washing conditions were as follows: 2 × SSC, 0.1% SDS at room temperature for 30 minutes and then at 60°C for 30 minutes, and finally in 0.1 × SSC, 0.1% SDS at 60°C for 1 hour. Occasionally, repeated hybridizations of a single nitrocellulose filter were carried out with different probes; in those cases, the bound probes were removed from the filter after each use by washing in TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0) at 68°C for 1 to 2 hours. The blots were exposed to Kodak XAR-5 film against two intensifying screens at −80°C for 3 to 4 days.

RESULTS

Absence of δ glycophorin in S-s-U- erythrocyte membranes. Immunoblots show that the erythrocyte membranes of both S-s-U- individuals lack the δ glycophorin monomer and dimer bands (3b and 6) when compared with a normal control (Fig 2A). These bands are readily identified in the S+s+U+ control erythrocytes by reaction with antigaicophorin serum and lack of reaction with antipeptide C serum (Fig 2A and B). The absence of δ glycophorin characteristically located in the PAS 3 band (2) is also seen in membrane preparations stained with PAS reagent (Fig 2C).
RESULTS

Serologic Characterization

The results obtained with sera from the three original patients with psuedoathrombocytopenia investigated in the PSIFT and the IFT on lymphocytes and granulocytes, at 20°C, are summarized in Table 1. The sera gave a positive reaction, both with the patients’ own platelets and with donor platelets; whereas no reaction was seen with donor granulocytes or donor lymphocytes, even in the presence of EDTA. When the indirect PSIFT was carried out with citrate platelets that had been washed and resuspended in PBS, no reaction occurred in two sera. In one serum (no. 3), citrate platelets also gave a positive reaction, although it was weaker. This latter reaction was stronger at 4°C. This result indicated that in this serum, in addition to EDTA-dependent antibodies, EDTA-independent cold antibodies against platelets were present. These EDTA-independent antibodies also reacted with platelets from heparinized blood, but the EDTA-dependent antibodies did not when no EDTA was added.

To prove the presence of separate cold antibodies, absorption and elution experiments were carried out. The sera from all three patients were absorbed with EDTA platelets or citrate platelets at 4°C; and from the absorbing platelets, ether eluates were prepared. The results are shown in Table 2. The sera absorbed with EDTA platelets showed no reactivity in the PSIFT against EDTA platelets. The reaction of the absorbed serum of patient Ve (no. 3) with citrate platelets also became negative. However, when this serum was absorbed with citrate platelets, a positive reaction against EDTA platelets remained, whereas the reaction against citrate platelets had disappeared. Eluates from the EDTA platelets showed a positive reaction with EDTA platelets, but not with citrate platelets in the first two patients. With the third serum, the eluates from both EDTA and citrate platelets showed a positive reaction with EDTA platelets as well as with citrate platelets.

EDTA Dependency of the Reaction

The two sera were studied that contained only EDTA-dependent antibodies. The sera gave a positive
the δ glycoprotein gene, but the 11.7-kb fragment is one of a pair of RFLP alleles (the other one being the 7.3-kb fragment) linked to the α N glycoprotein gene. The 14.2-kb fragment is analogous to the SacI 8.0-kb fragment in that it hybridized strongly with probe III but weakly with probe IV (Table 1). The 14.2-kb MspI fragment most likely encompasses the total coding sequence of δ glycoprotein gene. This is because the single cutting site for MspI, within the coding region of α glycoprotein gene may be absent in δ glycoprotein gene due to a different codon specifying δ glycoprotein at position 29. This residue in δ glycoprotein is the position of the δS/δs allelic substitution, Thr and Met in δs and δS, respectively, and is aligned with Thr 58 (ACC), in α glycoprotein. Assuming a single base change between the codons, it is likely that the codon for Thr-29 in δ glycoprotein would be ACG, given the unique codon for Met, ATG. Such an ACG codon would indeed eliminate the MspI site. This conclusion is supported by our failure to differentiate SS, ss or Ss genotypes using MspI.39

DISCUSSION

In this report, we provide evidence that two unrelated individuals who exhibit the S−s−U− blood group phenotype lack erythrocyte δ glycoprotein and that the absence of this protein correlates with deletion of the δ glycoprotein gene. This is the first documentation of a glycoprotein gene deletion. This was concluded primarily from comparative restriction mapping of genomic DNA from control and variant donors using as probes previously cloned cDNAs and probes derived from different regions of these DNAs that encode defined amino and carboxyl terminal regions of α and δ glycoproteins. Specific fragments were absent in five different enzyme digests of S−s−U− DNA, that in control DNA hybridized with the 5' or 3' terminal probes or with both. The conclusion that the fragments missing in the variant individuals derived from δ glycoprotein gene was inferred primarily from our ability to relate, in controls, the relative intensities of bands in given digests to regions of high and low homology in the amino acid sequences of α and δ
glycophorins. This allowed us to assign fragments as originating from the \( \alpha \) or the \( \delta \) glycophorin gene.

This study complements previous findings\(^1\) and results in a more conclusive identification of the gene origin of restriction fragments. This became possible not only by comparison of control and variant maps, but also through the use of cDNA fragment probes. In contrast to previously used restriction fragments for control and variant maps, but also through the use of more conclusive identification of the gene origin of restriction fragments observed in S + s + U + individuals: Hybridization of Restriction Fragments with cDNA Probes.

<table>
<thead>
<tr>
<th>Restriction Enzymes</th>
<th>Size (kb)</th>
<th>Control DNA</th>
<th>S - s - U - Variant DNA</th>
<th>α Terminal Specific</th>
<th>( \delta ) Terminal Specific</th>
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<tr>
<td>HindIII</td>
<td>7.4</td>
<td>++ + + + +</td>
<td>+ + + + + + + + + + + + + +</td>
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<tr>
<td>SacI</td>
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<td>++ + + + + + + + + + + + + +</td>
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</tr>
<tr>
<td>MspI(^\circ)</td>
<td>14.2</td>
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<td>++ + + + + + + + + + + + + +</td>
<td>++ + + + + + + + + + + + + +</td>
<td></td>
</tr>
</tbody>
</table>

Probes described in legend to Fig. 18.

\(^*\)Control DNA strongly with the 5' probe and the other with much less intensity with the 3' probe. Although the sizes of the deleted fragments suggest that the entire \( \delta \) glycophorin gene is deleted, using the cDNA probes we cannot now define the total size or the 5' and 3' endpoints of the deletion.

It became clear in the course of these studies that the restriction fragments observed in S + s + U + individuals originated exclusively from \( \alpha \) glycophorin. The patterns shown by these fragments and their assignment to the regions of \( \alpha \) glycophorin they encode are similar to those noted previously.\(^2\) In addition to these observations, however, the use of the 5' and 3' terminus-specific cDNA probes revealed in several digests two fragments that originate from the amino terminus of \( \alpha \) glycophorin gene and in the SacI digests two carboxy terminus-specific fragments. Our data indicate at least two exons in \( \delta \) glycophorin gene and suggest that the genomic organization of \( \alpha \) glycophorin gene may be more complex.

The S + s + U + phenotype of erythrocytes of the two individuals examined here correlates with the lack of \( \delta \) glycophorin in membranes of these cells; the demonstration that the \( \delta \) glycophorin gene is deleted from both chromosomes in each individual provides proof that these phenotypes correlate with the genotypes of the donors: donor M.E.K., \( \alpha N \delta^A/\alpha N \delta^S \); and donor M.L.A., \( \alpha M \delta^M/\alpha M \delta^S \). Such a straightforward relationship may not necessarily be observed in all cases. Other mechanisms, including defects in transcriptional, translational, or posttranslational processing may be operative in other individuals of the S + s + U + phenotype.

The possibility of alternative origins for the phenotype was recently revealed through a study of a Dantu blood group variant.\(^3\) As shown, erythrocytes of an individual exhibiting MN, Dantu, s + phenotype lacked \( \delta \) glycophorin, despite the s + phenotype. We have explained this apparent inconsistency by demonstrating that the Dantu variant glycophorin is a hybrid \( \delta-\alpha \) structure, which evidently retains the s specific antigenic determinant in the \( \delta \) glycophorin portion of the molecule. It will be of interest to determine if the \( \delta \) glycophorin gene is deleted in Dantu individuals. The complexity of the glycophorin gene described here and further implied by our previous studies as well as by the presence of many serologically identified blood group variants, suggests that the glycophorin gene locus will be particularly informative in studies of human gene recombination.

Gene deletion is a common mechanism underlying some human genetic disorders, most notably thalassemias.\(^4\) Certain thalassemias occur in geographically defined groups of individuals, for which it has been hypothesized that the absence of \( \alpha \) or \( \beta \) globin gene may have represented a selective advantage. It is therefore of interest that the S + s + U + blood group phenotype is prevalent among selected populations, in particular among Blacks from certain regions in Africa where the incidence of this phenotype is 1% to 35%\(^5\) as compared with an incidence of \(<0.001\% \) in the white population. Our current studies are thus an important first step in understanding of the origin of the S + s + U + phenotype.
always characterized by the δ glycophorin gene deletion and whether when the deletion occurs a single origin or multiple evolutionary origins are implicated.

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