A Point Mutation in the GYPC Gene Results in the Expression of the Blood Group An* Antigen on Glycophorin D But Not on Glycophorin C: Further Evidence That Glycophorin D Is a Product of the GYPC Gene

By Geoff Daniels, May-Jean King, Neil D. Avent, Ghizala Khalid, Marion Reid, Gary Mallinson, Jon Synthe, and Bertil Cedergren

Glycophorin C (GPC) and glycophorin D (GPD) are homologous sialoglycoproteins in the human red blood cell membrane. Both are thought to be encoded by the GPC gene (GYPC). We report that the rare blood group antigen, An*, is expressed on GPD but not on GPC. cDNA was synthesized from total RNA obtained from two unrelated, heterozygous An*+ blood donors and analyzed by the polymerase chain reaction using primers that spanned sequences encoded by the GYPC gene. The expected 412-bp fragment was generated, and sequencing of the amplified product showed a G → T substitution at nucleotide 67 of the coding sequence, resulting in the substitution of alanine by serine at amino acid residue 23 of GPC and, presumably, residue 2 of GPD. To explain the expression of An* on GPD but not on GPC, we postulate that the conformation of the amino acid residues at the N-terminal region of GPD determines the antigenic expression as this conformation would be different from that of the same sequence of amino acids occurring within GPC. Other possible reasons for antigen expression on a shorter protein product but not on the full-length protein product of the same gene are discussed. We extrapolate this reasoning to account for the expression of the common GE2 blood group antigen on GPD but not on GPC.

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the C-terminal residues 112 to 128 of GPC. This antibody recognizes determinants common to the C-terminal domains of GPC and GPD. Human anti-An\(^{+}\), anti-GE2, and anti-GE3 were from our collections. Polyspecific antihuman globulin reagent was obtained from Bio Products Laboratory (Diagnostics, Elstree, UK).

**Immunochemical methods.** Immunoblotting and immunoprecipitation were performed as described previously. For immunoblotting, RBC membranes were solubilized in the presence of SDS and mercaptoethanol, and the components separated by SDS-PAGE on 10% polyacrylamide gels with a 3% stacking gel. The proteins were then electrophoresed onto polyvinylidene difluoride filters (Millipore, Watford, UK), incubated with MoAb culture supernatant, human antibody (prepared by adsorption onto and elution from antigen positive RBCs), or diluted rabbit antibody, and detected using, respectively, horseradish peroxidase conjugated antihuman IgG, antihuman Igs, or antirabbit Igs (Dako, High Wycombe, UK), with 4-chloro-1-naphthol as substrate.

For immunoprecipitation, rabbit beta serum was incubated with RBC membranes. These sensitized RBC membranes were solubilized in 1% Triton X-100 in phosphate-buffered saline (PBS) and antibody-antigen complexes precipitated with protein A Sepharose (Pharmacia, Milton-Keynes, UK). Isolated material was analyzed by SDS-PAGE and immunoblotting.

**Serologic methods.** Standard tube techniques with untreated or enzyme-treated RBCs were used throughout. Antiglobulin tests were read after centrifugation. Titration were performed by using doubling dilutions of serum or MoAb culture supernatant followed by addition of a constant volume of a 5% saline suspension of RBCs. Titration scores were the sum of scores assigned to each dilution according to the strength of reaction (+ + + + + = 12, ++ + + = 10, ++ + = 8, ++ = 5, (+) = 3, w = 2). RBCs were pretreated with papain, trypsin, α-chymotrypsin, pronase, or sialidase as described previously. For immunoprecipitation, rabbit beta serum was incubated with RBC membranes. These sensitized RBC membranes were solubilized in 1% Triton X-100 in phosphate-buffered saline (PBS) and antibody-antigen complexes precipitated with protein A Sepharose (Pharmacia, Milton-Keynes, UK). Isolated material was analyzed by SDS-PAGE and immunoblotting.

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The following synthetic peptides were prepared by Research Genetics (Huntsville, AL) and used as inhibitors: H\_2N-M-A-S-S-T-T-M-H-T-COOH (D1-10) and H\_2N-M-S-S-A-S-T-T-M-H-T-COOH (ANAD). Peptide inhibition were performed by mixing equal volumes of 1 mg/mL synthetic peptide in PBS and suitably diluted anti-An\(^{+}\) or anti-GE2 serum, incubating for 40 minutes, and using the mixture in an indirect hemagglutination test.

**Isolation of total RNA.** Total RNA was isolated from EBV-transformed cell line from An\(^{+}\) donor BM using an RNA extraction kit (Pharmacia). Total RNA from An\(^{+}\) donor MS and an An\(^{+}\) individual were isolated from a reticulocyte-rich fraction of whole blood using the method of Temple et al.

**Amplification of glycophorin C-related cDNA by polymerase chain reaction (PCR).** The method for synthesis of cDNA templates was adapted from Ausubel et al. In brief, 6 μg of total RNA was incubated with 0.25 mMol/L dNTP, 10 μg oligo-dT\(_{18}\), 39 U RNA guard, 50 U AMV reverse transcriptase (all obtained from Pharmacia in 10 mMol/L DTT, 50 mMol/L KCl, 6 mMol/L MgCl\(_2\), and 50 mMol/L Tris-HCl buffer, pH 8.2, for 1.5 hours at 42°C. The incubation was terminated by phenol extraction. cDNA were precipitated by ethanol and then resuspended in a final volume of 50 μL TE buffer (10 mMol/L Tris, 1 mMol/L EDTA) (pH 7.6).

PCR amplification of cDNA templates were performed in reaction mixtures of 50 μL final volume: 1 μL of the cDNA template preparation, 0.25 mMol/L each dNTP, 50 mMol/L KCl, 2 mMol/L MgCl\(_2\), 0.4 mMol/L each primer, 10 mMol/L Tris-HCl (pH 8.4), and 1.25 U Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT). The sense primer (C5) had the sequence 5'-TCCCCGCGGTCTCTGCCCGG-3', and the antisense primer (NC3) had the sequence 5'-GTACTCTTTTCGCTGATACCC-3' and corresponded, respectively, to nucleotides -34 to -15 and 378 to 355 in the GPC cDNA sequence. Amplification was performed by PCR for 35 cycles on a Perkin-Elmer/Cetus thermal cycler using the following conditions: denaturation for 1 minute at 94°C, annealing for 20 seconds at 69°C, and extension for 1 minute at 72°C. The PCR product was purified on a 5% polyacrylamide gel and cloned into EcoRV cut phosphatase-treated Bluescript phagemid (Strategene cloning systems, La Jolla, CA) as previously described.

**DNA sequencing.** Both strands of subcloned PCR products were sequenced after alkali-denaturation by dyeodeoxy chain termination method using sequenase (US Biochemicals, Cleveland, OH). Sequencing of the entire 412 nucleotides from both strands of GPC-related cDNA was performed using synthetic oligonucleotides (NC3, C5, C2, and β1) as primers. The sequence of C2 was 5'-ACATGCATACTACCGCATGC-3' (nucleotides 82 to 104), and the sequence of β1 was 5'-CTGCAATGACGCAATGTCGAC-3' (nucleotides 190 to 172). Products of the sequencing reaction mixtures were separated on buffer gradient gels.

**RESULTS**

**Detection of An\(^{+}\) on GPD.** Immunoblotting of RBC membranes from three unrelated An\(^{+}\) individuals with eluates prepared from two sera containing anti-An\(^{+}\) showed that the An\(^{+}\) antigen was located on a component of M, 30,000 (Fig 1, tracks b through d), which had the same mobility as that determined for GPD using anti-GE2 (Fig 1, tracks c through e). This component was not detected with anti-An\(^{+}\) on blots of membranes prepared from An\(^{+}\) RBCs (Fig 1, track a).

Probing immunoblots with human anti-GE2, anti-GE3 (data not shown), MoAb GERO, and the rabbit beta serum did not show any difference in staining intensity or mobility of GPC or GPD from An\(^{+}\) or An\(^{+}\) RBCs (Fig 1, tracks e through h). To confirm that the An\(^{+}\) antigen is located on GPD, immunoprecipitation of GPC and GPD from An\(^{+}\) RBCs was performed using the rabbit beta serum, followed by immunoblotting of the immunoprecipitate with one human anti-An\(^{+}\). Only the GPD in the immunoprecipitate reacted with anti-An\(^{+}\) (Fig 1, track m).

**Serologic comparison of An\(^{+}\) and GE2 antigens.** The four An\(^{+}\) individuals were presumed to be heterozygous for the gene controlling An\(^{+}\) expression. Their An\(^{+}\) RBCs have the common Gerich phenotype GE: 2, 3, 4. Since GE2 and An\(^{+}\) antigens are expressed exclusively on GPD, we examined whether the presence of An\(^{+}\) affected the expression of GE2 antigens. Hemagglutination titration studies were performed using 13 anti-GE2 on four An\(^{+}\) RBC samples. While eight antisera gave no difference in titration score, five reacted more weakly with An\(^{+}\) RBCs when compared with the An\(^{+}\) controls (Table 1). Titrations with five human polyclonal anti-GE3 (which detect a determinant common to GPC and GPD) and with two human polyclonal and one mouse monoclonal anti-GE4 (which detect a determinant close to the N-terminus of GPC) showed no difference in strength between An\(^{+}\) and An\(^{+}\) RBCs.

Anti-An\(^{+}\) resemble most anti-GE2 in their reactions with RBCs treated with various enzymes. Anti-An\(^{+}\) (2 examples) and anti-GE2 either did not react or reacted only weakly...
with antigen-positive RBCs treated with trypsin, α-chymotrypsin, or papain. Both anti-An* and most anti-GE2 did not react with sialidase treated RBCs, showing that An*, like GE2 as determined by the majority of anti-GE2, is a sialic acid dependent determinant.

RBCs were treated with acetic anhydride to acetylate free amino groups. MoAb GERO, which detects an epitope at the N-terminus of GPC, did not agglutinate acetylated RBCs, demonstrating the efficacy of blocking free amino groups at the N-terminus of GPC. The reactivity of two anti-An* with acetic anhydride treated An*+ RBCs was indistinguishable from that with untreated An*+ RBCs (Table 1).

### Table 1. Titration Scores of Anti-GE2 and Anti-An* Sera With An*+ and An*- RBCs and With Acetylated RBCs

<table>
<thead>
<tr>
<th>Anti-GE2</th>
<th>An*+</th>
<th>An*</th>
<th>Acetylated BM</th>
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<tbody>
<tr>
<td>PL</td>
<td>0</td>
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</tr>
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<td>Iwe</td>
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<td>0</td>
</tr>
<tr>
<td>Ott</td>
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<table>
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<tr>
<td>Lin</td>
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Abbreviation: NT, not tested.

In similar experiments, two of 10 anti-GE2 failed to agglutinate acetic anhydride treated An*+ RBCs (Table 1).

**Sequencing of GPC-related cDNA.** First-strand cDNA, produced by reverse transcription of mRNA isolated from an Epstein Barr Virus (EBV)-transformed lymphoblastoid cell line from donor BM, was amplified by PCR using primers C5 and NC3. The expected 412-bp product was obtained from the An*+ control and also from the An*+ donor. Template controls did not produce any products after PCR amplification. Six independent clones from the PCR product from BM were sequenced from nucleotides 34 to 378. In two clones, a G → T substitution was detected at nucleotide 67 of the coding sequence of GPC cDNA (Fig 2). The expected C → A substitution on the antisense strand was confirmed. Sequencing of the remaining four cloned PCR products did not show any differences from the expected nucleotide sequence of the GPC cDNA. Thus, of the six clones analyzed, two were from the GYPc gene encoding the An* blood group (GYPc.AnP) and four were from the partner normal GYPc gene.

The association of An* with a single point mutation was confirmed in tests on a second, unrelated An*+ individual (MS). A single 412-bp PCR product was again obtained. The substitution at nucleotide 67 was shown to occur on both strands of cDNA prepared from total RNA isolated from reticulocytes in whole blood from MS. Of five clones analyzed, one was from the GYPc.AnP gene and four were from the partner normal GYPc gene.

**Inhibitions with synthetic peptides.** Synthetic peptides representing the N-terminal 10 amino acid residues of normal GPD and of GPD An*, as deduced from the GYPc.AnP nucleotide sequence, were used in hemagglutination inhibition experiments with three anti-An* (Berg, Lin, AB) against An*+ RBCs and two anti-GE2 (PL, ZY) against GE:2 An*+ and GE:2 An*+ RBCs. No inhibition was observed in any of the tests.
Both GPC and GPD are extensively O-glycosylated at their extracellular domains. The sialic acid dependency of the anti-An antigen reactivity indicates the involvement of sialylated O-glycans in the expression of the corresponding antigen. The alanine to serine substitution in An individuals creates a potential O-glycosylation site (Fig 3). Direct O-glycosylation of the substituted serine or the neighboring serine/threonine residues may provide the sialic acid requirement of the An antigen. However, immunoblotting showed no differences in M₆ of GPC and GPD from An⁺ RBCs and An⁻ RBCs, which would have been an indication for varying degrees of glycosylation. Whether the substituted serine is glycosylated or not, the localization of the An antigen to GPD. An still favors the postulation that the conformational differences at the N-terminal domain account for the lack of An expression on GPC. An⁺

The high incidence blood group antigen GE2 is the only other known immunologic marker expressed on GPD but not on GPC.⁴ Thus, the GE2 antigen may be at a similar location to the An antigen at the N-terminal domain of GPD. Therefore we compared the expression of GE2 on An⁺ and An⁻ RBCs by titration with several anti-GE2 sera. Anti-GE2 sera are characterized by their failure to react with RBCs from Gerbich negative individuals with deletions in either exon 2 (phenotype Ge: -2, 3, 4) or exon 3 (phenotype Ge: -2, -3, -4) in the GYP gene.⁵⁻⁷ RBCs from these Gerbich negative individuals express no GPD. Our results showed that anti-GE2 may represent a heterogeneous group of antibodies. Some anti-GE2 sera agglutinated An⁺ RBCs more weakly than An⁻ controls, suggesting that the presence of An reduces the expression of GE2 on An⁺ RBCs, at least as determined by some anti-GE2. This could be due to the absence of the GE2 antigen in an antithetical relationship with An. Some anti-GE2 reacted equally with An⁺ and An⁻ cells, suggesting that these antibodies do not discriminate between Ala as in normal GPD and Ser² as in GPD. An. In the four heterozygous individuals studied here, the presence of normal GPD together with GPD. An affects the expression of GE2 on An⁺ RBCs, at least as determined by some anti-GE2. This would mean that the cells would still react with anti-GE2 even if GPD. An were to express no GE2 antigen. The interpretation of these results would be more conclusive if an individual homozygous for the GYP.C. An gene was discovered, but such an individual would be extremely rare.

The possibility that the An⁺ antigen could be expressed on GPC. An if the N-terminal 21 amino acids were removed was considered, and protease cleavage sites (eg, protease V8) in the vicinity of Met² were of GPC were examined. Since no suitable sites were identified, we suggest that in vitro translation of GYP.C. An eRNA may be more revealing, with the probable expression of two products, GPC. An and GPD. An, in which only GPD. An carries the epitopes recognized by anti-An⁺.

Attempts to inhibit hemagglutination by anti-An⁺ or anti-GE2 with synthetic peptides D1-10 and ANAD representing, respectively, the deduced N-terminal 10 amino acid residues of GPD and GPD. An were unsuccessful. This re-
result was not surprising as RBC expression of An and GE2 is dependent on the presence of sialic acid.

In conclusion, the An phenotype is the result of a point mutation in the GYP gene (GYPAn), which probably encodes two glycoprotein products GPC.An and GPD.An. The An antigen is only expressed on GPD.An, presumably because of the proximity of the substituted amino acid to the N-terminal region of GPD.An. The localization of the An antigen to the N-terminus of GPD.An provides immunologic evidence for the model that GPD is encoded by the GYP gene, as suggested previously. This is possibly a further example of a highly unusual event in eukaryotic gene expression.

NOTE

These findings have elevated the An antigen from a low incidence antigen in the 700 series to being part of the Gerbich blood group system with the number 020007 (GE7).

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A point mutation in the GYPC gene results in the expression of the blood group Ana antigen on glycophorin D but not on glycophorin C: further evidence that glycophorin D is a product of the GYPC gene

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