Molecular Basis for the Human Erythrocyte Glycophorin Specifying the Miltenberger Class I (MiI) Phenotype

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Human glycophorin Mil (HGpMil) is a structural variant of the MNSs blood group system that specifies the Miltenberger class I phenotype. We report here the molecular basis of the HGpMil gene identified in a white family in which the first homozygote was encountered. Immunoblotting analysis showed the expression of HGpMil and HGpB but the absence of HGpA on the homozygous Mil erythrocytes. Southern blot analysis detected no gross alterations in gene structure or band intensity. Genomic sequences encompassing exons II and III of the HGpMil gene were amplified by single-copy polymerase chain reaction. Restriction digestion and direct DNA sequence analysis showed that HGpMil gene is derived from an α/α allele of HGpA and differs from the latter in the third exon by a single nucleotide change. In HGpMil, the presence of a deoxynzymidine at the second position of codon 28 (ATG) not only resulted in a methionine substitution but also altered the consensus sequence for N-glycosylation from Asn-Asp-Thr to Asn-Asp-Met. These data are consistent with the occurrence of Mil on the red blood cell membrane as a variant deficient in the asparagine-linked carbohydrate unit. Significantly, this particular point mutation lies in between the two half-sites of a direct repeat that has been implicated to facilitate the recombination events leading to several other glycophorin genes of the Miltenberger series. Based on this relatedness, we propose an untemplated nucleotide replacement resulting from a gene conversion event as the molecular basis for the origin of HGpMil gene.

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EXPERIMENTAL PROCEDURES

Blood samples. Peripheral bloods were obtained from Mil homozygote (propositus E.H.) and heterozygotes of a white family. The MNSs blood group phenotype of the propositus is M-, N+, S-, s+. The Mil phenotype was established by agglutination of her red blood cells with serum possessing anti-Vw specificity and lack of agglutination with anti-Mur, -Hil, -Hut, -Anek, -Raddon, and -Lane. A serologic and biochemical study of the Mil-carrying family, including the propositus, will be described elsewhere (P.S., J.J.M., in preparation). Peripheral bloods used as controls were obtained from normal family members or normal human blood donors.

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Western blot analysis of erythrocyte glycoporphins. Procedures previously described were used.\textsuperscript{19} Erythrocyte lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membrane. Glycoporphins were visualized by autoradiography after reaction with one polyclonal antiserum (anti-\(\alpha\)GP) and with monoclonal antibodies (MoAbs) specific for the M and N blood group antigens (M MoAb and N MoAb). Glycoporphins were examined for the presence of the N-linked carbohydrate unit by treatment with N-glycanase (Genzyme, Cambridge, MA), as described.\textsuperscript{20}

Southern blot analysis of genomic DNAs. High-molecular-weight genomic DNAs were isolated from the leukocyte nuclear pellets of the Mi1 homozygote and normal blood donors as described.\textsuperscript{21} Analysis of genomic DNA restriction digests by Southern blots\textsuperscript{22} was performed as described.\textsuperscript{23} The hybridization probes included one cDNA insert of HGP\(\alpha\)(\(\alpha\)M)\textsuperscript{24} and two intervening sequences, IVS 2 and IVS 3\textsuperscript{10}; they were labeled with [\(\alpha\)-\(\text{\textsuperscript{32}P}\)]dCTP (Arlington Heights, IL) by random primer extension.\textsuperscript{25}

Amplification of genomic DNA sequences by single-copy polymerase chain reaction (sc PCR). Genomic sequences of normal and variant glycoporphin genes were amplified by Taq DNA PCR.\textsuperscript{26} To generate gene-specific DNA segments for restriction and direct DNA sequence analysis, a modified procedure termed sc PCR\textsuperscript{27} was used. A single copy HindIII fragment (1.6 kb) unique to the HGP\(\alpha\) gene was recovered by electrophoresis and used as the PCR template to generate the genomic DNA segment encompassing exons II to III. Because the propositus was homozygous for the Mi1 phenotype no fragments from HGP\(\alpha\) gene were expected to be present. As controls, the HGP\(\alpha\) 1.6 kb (HindIII) and HGPB 8.0 kb (SacI) fragments were recovered from a normal donor homozygous for the M phenotype and used as PCR templates. Amplification primers were synthesized on a 380A DNA synthesizer (Applied Biosystems, Foster City, CA) and purified by 15% polyacrylamide/7.0 mol/L urea gel electrophoresis; M1-8 and N1-8 were 24-mer forward primers that define codons 1 through 8 for the M and N blood antigens, respectively, whereas IVS3R was a 25-mer reverse primer that spans 61 to 85 bp downstream from the first nucleotide of intron 3. The sequences of M1-8, N1-8, and IVS3R are, respectively, 5'-CGATGTCACCTGAGGTGGC-3', 5'-AGAACTGTCATGTTACAAGCTCGT-3', and 5'-AGATCAGCTGCGT-3'. M1-8 and IVS3R were used to amplify HGP\(\alpha\) and HGPB gene segments, whereas M1-8 and IVS3R were used to amplify HGP\(\alpha\) gene segments. Conditions for PCR cycling in a single block thermocycler (Eppendorf, San Diego, CA) were as described previously.\textsuperscript{10} Aliquots of PCR products or their restriction digests were analyzed by electrophoresis on 1.6% agarose or 5% polyacrylamide gels.

Direct DNA sequencing. The PCR-amplified double-stranded DNA fragments were directly sequenced by the dideoxy chain termination procedure\textsuperscript{27} using the Taq DNA polymerase (Sequenase, United States Biochemicals, Cleveland, OH) and 5' end-labeled synthetic primers. The PCR products were extracted with chloroform, precipitated with ethanol, and freed of residual dNTP and amplimers by native 5% PAGE. The template DNA was cut out from the gel under UV light shadowing, eluted in TE buffer for 8 to 10 hours at 37°C, and recovered by centrifugation. Before use all of the sequencing primers were purified by 15% polyacrylamide/7.0 mol/L urea gel electrophoresis, labeled at 5' ends with [\(\gamma\)-\(\text{\textsuperscript{32}P}\)]ATP (DuPont-New England Nuclear, Boston, MA) by T4 polynucleotide kinase, and spin-dialed in Centricon 10 (Amicon, Beverly, MA). TE-eluted template DNA, 0.5 to 1 pmol, was hypophosphor and reconstituted in a 12.5-\(\mu\)L volume containing 2 \(\mu\)L 5X reaction buffer and 4 to 5 pmol of \(\text{\textsuperscript{32}P}\)-primer. The conditions for sequencing reactions and polyacrylamide-urea gel electrophoresis were the same as described previously.\textsuperscript{5,7,10}

RESULTS

Identification of glycoporphin Mi1 by Western blotting. Figure 1 illustrates the Western blot profiles of erythrocyte glycoporphins from the Mi1 homozygote and one heterozygote. Glycoporphin Mi1 can be readily identified by its specific MoAb demonstrated that it is associated with the heterodimer of red blood cell lysates fractionated by SDS-10% polyacrylamide gels were visualized by an anti glycoporphin antiserum (anti-\(\alpha\)GP, A) and an N blood group-specific MoAb (N MoAb, B), respectively. In (C), isolated glycoporphins were treated with (+) and without (−) \(\alpha\)-glycanase and probed with anti-\(\alpha\)GP, \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) glycoporphin dimer and monomer; \(V_2\), \(V_1\), Mi glycoporphin dimer and monomer (also shown by arrows). The band of approximately 80 Kd in Mi1 lanes is a heterodimer \(\alpha_1\)\(\alpha_2\). Molecular weight markers are shown at left margin.

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**Fig 1. Western blot analysis of erythrocyte glycoporphins from Mi1 family members.** 1, Control; 2, Mi1 heterozygote; 3, Mi1 homozygote. Immunoblots of red blood cell lysates fractionated by SDS-10% polyacrylamide gels were visualized by an anti glycoporphin antiserum (anti-\(\alpha\)GP, A) and an N blood group-specific MoAb (N MoAb, B), respectively. In (C), isolated glycoporphins were treated with (+) and without (−) \(\alpha\)-glycanase and probed with anti-\(\alpha\)GP, \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) glycoporphin dimer and monomer; \(V_2\), \(V_1\), Mi glycoporphin dimer and monomer (also shown by arrows). The band of approximately 80 Kd in Mi1 lanes is a heterodimer \(\alpha_1\)\(\alpha_2\). Molecular weight markers are shown at left margin.
treatment (Fig 1C). This indicates that the increased mobility of Mi1 glycophorin must be caused by the lack of a single asparagine-linked carbohydrate unit.

**Analysis of Mi1 genomic DNA by Southern blotting.** Genomic DNAs from both Mi1 and normal individuals were examined by restriction endonuclease mapping for alteration in the glycophorin genes. No gross DNA rearrangement or deletion was detected with cDNA or genomic DNA probes after restriction digestion by HindIII and several other enzymes (not shown). These data indicate that the variability in the Mi1 homozygote may result from a point mutation or minor alteration in the coding region of HGpMi1 gene. In particular, the codons 26 through 28 spanning the exon II/exon III junction could be involved as indicated by the abolishment of the N-glycosylation site.

**Amplification and analysis of genomic sequences from the HGpMi1 gene.** Figure 2A illustrates the specific amplification of a 0.97-kb segment from the 1.6-kb HindIII restriction fragment of the HGpMi1 gene. This segment should encompass exon II through exon III of the HGpMi1 gene. As shown, a single band comparable with the HGpA and HGpB gene segments in size is observed in agarose gel electrophoresis (Fig 2B). Because the MspI and SmaI restriction sites in this region display asymmetrical distribution around the HGpA and HGpB genes, the amplified DNA segments were digested with the two enzymes to verify their nature and origin. The presence of MspI and SmaI sites in HGpMi1 and HGpA, but not in the HGpB gene segment (Fig 2C), demonstrates that the HGpMi1 and HGpA genes are closely related.

**Nucleotide sequence of the HGpMi1 gene.** The nucleotide sequence spanning exons II through III of the HGpMi1 gene was obtained from direct sequencing of the amplified 0.97-kb DNA segment. This segment was sequenced in its entirety; the exons and exon-intron junctional regions were sequenced twice on both strands. The DNA sequence...
analysis showed that the HGPmpl gene is HGPp(a)-like bearing a critical nucleotide change in its third exon, marked as being different from the HGPpA and HGPpB genes.

Figure 3 shows the DNA sequence ladders spanning the 3' end of intron 2 and the 5' end of exon III in the HGPpA, HGPpB, and HGPmpl genes. Figure 4 illustrates the nucleotide sequence encompassing exons II and III and the deduced amino acid sequence from residues 1 to 58 of MiI glycophorin. In the region spanning nucleotides 759 through 814, HGPmpl is identical with HGPpA except for a difference at nucleotide 790 where a deoxycytidine to deoxythymidine (C-T) transition has taken place (arrow). This nucleotide change is located at the second position of codon 28 and is expected to introduce a methionine into the MiI protein. When compared with HGPpB, the HGPmpl gene bears three nucleotide differences between nucleotides 759 and 814, including an A→T transversion at nucleotide 790 (arrow). The C versus A versus T substitution at the same nucleotide position suggests an evolutionary relatedness of the three glycoporphin genes in the process of diversification.

Further inspection of the intron 2/exon III junction shows the occurrence of a direct repeat of the acceptor splice site in tandem arrangement (Fig 4). This repeat is perfect in HGPpA and HGPmpl but imperfect in HGPpB because of a single G→C transversion at nucleotide 810 or the fifth position of the downstream half-site. The genomic sequences immediately adjacent to the direct repeat exhibit strong strand asymmetry. The 5' side of the upstream half-site is a pyrimidine-rich sequence that constitutes part of the structural elements necessary for splicing, whereas the 5' side of the downstream half-site is relatively rich in purine nucleotides. This local asymmetry may have been an important factor in generating the direct repeat as an initiation site for gene conversion events resulting in HGPmplX and MiI genes and it may have also contributed to the segmental transfer leading to HGPmplIII and MiVI genes. Notably, the nucleotide change found in HGPmpl
gene resides in between the two half-sites of the direct repeat. These observations implicate a possible role of DNA recombination in the origin of HGpMiI gene.

The DNA sequence (Fig 4) also shows that HGpMiI gene retains in exon II a sequence defining the N blood group antigen, consistent with the results of immunoblotting analysis (Fig 1B) and DNA typing (not shown). With regard to the intron 2 sequence, the HGpMiI gene bears those nucleotide substitutions characteristic of HGpA rather than the HGpB gene. These observations clearly relate this HGpMiI gene to a HGpA(aN) gene. In the HGpA gene, codons 26 through 28 (AATGATACG) span the exon II and exon III junctions and specify Asn-Asp-Thr, the only consensus sequence present in HGpA\(^9\) for attachment of carbohydrate chain at asparagine 26 (Fig 4). Therefore, the C-T replacement at codon 28 not only leads to a Thr to Met substitution but also alters the coding sequence that directs the N-glycosylation.

**DISCUSSION**

MiI is a variant phenotype of the MNSs blood group system recognized by its reaction with anti-Vw, one of a set of antibodies specific for the Miltenberger complex.\(^{30}\) Protein sequencing of a portion of the extracellular domain had indicated that MiI glycophorin was a variant of HGpA with a Thr→Met substitution at amino acid residue 28.\(^{17}\) Accordingly, this amino acid exchange resulted in the loss of N-glycosylation of the adjoining Asn at position 26.

The molecular analysis of the HGpMiI gene is in full agreement with the above findings. In the genomic DNA of the MiI homozygote examined here, a normal complement of HGpB and HGpE genes was present but the HGpA gene was replaced by the HGpMiI gene. The only alteration detected in the HGpMiI gene, when compared with the corresponding positions in HGpA and HGpB, was an untemplated thymidyl nucleotide mutation in exon III occurring 4 bp downstream from the last nucleotide of intron 2. This single nucleotide change resulted in the expression of the MiI protein bearing a Met at position 28 and lacking the usual N-linked carbohydrate unit.

Although a spontaneous point mutation would be consistent with such an alteration (Fig 5A), other molecular events, particularly those via gene conversion, can provide a more compelling explanation for the origin of HGpMiI gene. We have shown that glycophorin variants frequently occur in hybrid forms that may have originated from DNA rearrangements through gene conversion events.\(^{10-12}\) In particular, MiIII, MiVI, MiIX, and MiX genes are hybrids resulting from replacements of a segment in one parent gene by the homologous segment of the other parent. The minimal sizes of the transferred sequence from HGpA[a] to HGpB[8] or vice versa vary from 16 to 130 nucleotides in these four genes and the α-θ or δ-α breakpoinnts are flanked by direct or inverted repeats in α exon III or δ pseudoexon, a region previously identified as a hot spot of recombination.\(^{10}\) We propose that similar gene conversion events occurring in this region but involving a single untemplated nucleotide replacement, could have resulted in MiI gene. Our reasons are as follows: (1) The position corresponding to nucleotide 790 where the substitution is seen in MiI is marked as being different in HGpA and HGpB. (2) The proximal gene conversion breakpoints identified in MiIX and MiX genes are confined to a direct repeat at the intron 2/exon III junction.\(^{11,12}\) Significantly, the C versus A versus T replacement at nucleotide 790 is also flanked by the two half-sites of the same repeat (Fig 4). (3) The single nucleotide replacements associated with gene conversion events may not be rare in the glycophorin gene system as exemplified by the occurrence of an untemplated adenyl nucleotide mutation in the breakpoint region of HGpMiIX gene.\(^{12}\) (4) The genomic region spanning nucleotides 759 through 814 is frequently involved in the homologous recombination between the HGpA and HGpB genes.

Figure 5B presents a hypothetical scheme that explains the possible origin of HGpMiI gene. In this model, a segment of the HGpB gene spanning the intron 2/pseudoexon III junction may have replaced the homologous counterpart of the HGpA gene by strand invasion, branch migration, and heteroduplex formation. The resulting heteroduplex region encompassed by nucleotides 759 and 810 should retain a single A:C mismatch at nucleotide 790. The subsequent repair synthesis and resolution of the α-δ and δ-α junction points defined by nucleotides 759 through 790 and 790 through 810, respectively, could have converted the intermediate into two kinds of variant glycophorin genes, one templated and the other untemplated. One can predict
that if the invading strand of HGpB is used as a DNA template, a single templated adenyl nucleotide replacement would occur, resulting in an AAG codon for lysine at position 28 (Fig 5B, left); such a gene should encode MiII glycoporin. However, if the template-directed mismatch repair has failed, a thymidyl nucleotide then can be introduced “spontaneously” into this position leading to an ATG codon for methionine in MiI glycoporin (Fig 5B, right). As shown in Fig 4, the nucleotide sequences of the HGpMiI gene upstream from nucleotide 759 and downstream from nucleotide 810 are uniformly HGpA-like. Therefore, had the gene conversion event occurred, the segmental replacement would range in length from one (minimum) to 50 nucleotides (maximum).

Single nucleotide replacements possibly resulting from gene conversion events have been described in the Ig and major histocompatibility complex (MHC) gene systems. They have provided an additional means for antibody diversification or antigenic variation in the MHC allelic forms. Earlier work also has shown that introduction of homologous DNA sequences into a cell may result in untemplated mutation in the cognate gene, probably because of incorrect repair of the heteroduplex formed between the two recombining sequences. In light of the possibility that heteroduplex repair can induce mutations, our hypothesis concerning the origin of the HGpMiI gene from gene conversion-like event provides a plausible mechanism for the templated (predictable) and untemplated (unpredictable) nucleotide replacements that have occurred in the diversification of human glycophorins. Furthermore, the proposed gene conversion scheme for HGpMiI gene (Fig 5B) conforms with the unified view for the molecular basis of HGpMiII, MiIX, and MiX genes.

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