Synthetic Peptides Homologous to Human Glycophorins of the Miltenberger Complex of Variants of MNSs Blood Group System Specify the Epitopes for Hil, S^d, Hop, and Mur Antisera

By K.K. Johe, V. Vengelen-Tyler, R. Leger, and O.O. Blumenfeld

The antigenic epitopes of the MNSs blood groups are localized on α and δ glycophorins (glycophorins A and B) of the erythrocyte surface. Hil, S^d, Mur, and Hop antisera define the Miltenberger (Mi) complex of MiV, MiJ.L., MiIII, and MiVI variant serologic phenotypes of this blood group system. We report here the location of the epitopes for antibodies in these antisera. The antigens of these Mi classes are variant glycophorins that are hybrids of α and δ glycophorins in α-δ and δ-α-δ arrangements. The hybrid junctions give rise to novel polypeptide sequences not present in the parent glycophorins. In MiIII and MiVI this also includes an expressed sequence of the δ pseudoexon. These sequences are identical in the above Mi-glycophorins occurring in erythrocytes that share a common Mi determinant. Four peptides of 10 to 14 amino acids each were constructed to be homologous to the identical sequences; they were designated, "Hil", "S^d", "Mur", and "Hop" to reflect the common determinant. The peptides were tested for inhibition of reaction of appropriate cells with the relevant antisera. The Hil peptide, outlining the α-δ junction region in MiIII, MiV, and MiVI glycophorins, inhibited the reaction of respective erythrocytes (red blood cells [RBCs]) with anti-Hil. The S^d peptide, which differs from the Hil peptide by a single Thr → Met substitution, was specific for inhibition of the reaction of MiJ.L. RBCs with anti-S^d (an example of anti-S specific for such RBCs). The Hop peptide, which corresponds to the δ-α junction in MiVI glycophorin, inhibited the hemagglutination of MiVIII RBCs by anti-Hop. MiVI and MiVIII glycophorins share an identical sequence at that site. The Mur peptide, corresponding to a portion of the expressed pseudoexon sequence in MiIII and MiVI glycophorins, was specific for inhibition of the reaction of MiIII and MiVI RBCs with anti-Mur. The peptides had no effect on the hemagglutination of control MNSs RBCs by their respective antisera nor of unrelated Mi classes RBCs by antisera that distinguish these classes. We conclude that the α-δ junction in MiIII, MiV, and MiVI glycophorins outlines the epitopes for anti-Hil, the α-δ junction in MiJ.L. outlines the epitope for anti-S^d, the δ-α junction in MiVI constitutes the epitope for anti-Hop, and the expressed δ pseudoexon sequence in MiIII and MiVI constitutes the epitope for anti-Mur.

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Structures of MiIII and MiJ.L. glycophorins were elucidated by direct protein sequencing, 6,5 and those of MiV and MiVI were deduced from the structures of their genes. 13,14 The gene structures of MiIII and MiJ.L. have also been established recently and the directly determined and deduced protein sequences are in full agreement. 12,13 All four of these variant glycophorins are hybrids of α- and δ-glycophorins, with no apparent extraneous sequences (Fig 1). MiV and MiJ.L. are α-δ hybrids differing only in the form of the δ allele that is inferred to have participated in hybrid formation 12,15,16; thus, MiV is an α^d-δ hybrid and MiJ.L. is an α^d-δ hybrid and the two proteins differ by a single residue (Thr4 in MiV and Met14 in MiJ.L., Table 1). MiIII and MiVI are nearly identical δ-α-δ hybrids that differ by a single amino acid residue at position 48 (Arg in MiIII → Thr in MiVI) 13 (Table 1). The structures of the latter two glycophorins are closely related to δ-glycophorin 7 except that they contain inserts of an expressed segment of a δ pseudoexon sequence (an intron sequence in the δ gene homologous to α exon 3) and a relatively short stretch of δ-glycophorin residues (Fig 1).2,13 Whereas in δ-glycophorin the pseudoexon sequence is not expressed due to an alteration at a potential 5' consensus splicing signal,1 parts of this sequence are expressed in MiIII and MiVI glycophorins because, as a result of gene conversion, the functional splicing signal of the α gene is introduced into MiIII and MiVI genes.6,13

In all the hybrid glycophorins, the α-δ or δ-α junction regions and the expressed pseudoexon sequence constitute "new" polypeptide sequences that are not present in the parent α- and δ-glycophorins. Inspection of these "new" sequences showed that they are shared by several Mi-glycophorins (Table 1, underlined), and a close correspondence was found between the common sequences and the reactions of the relevant RBCs with specific antisera. For...
Peptides were desalted on Sephadex G-10 equilibrated with 10% for elution? The composition of the major peaks was verified by Applied Biosystems, Foster City, CA) and standard programs.

**EPITOPES OF MILTENBERGER ANTIGENS**

**Table 1. Structural Alterations and Common "New" Sequences**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Variant Glycophorine</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNDTHK</td>
<td>RDTYAATPRA HEVSEISVRT VYPPEEETGE</td>
<td>2</td>
</tr>
<tr>
<td>TNDkhk</td>
<td>r d t y p a h t a n e v s e i s v t t v s p e k k n G E</td>
<td>3</td>
</tr>
<tr>
<td>HGPMIV</td>
<td>TGQLVRHFTVP</td>
<td>12</td>
</tr>
<tr>
<td>HGPMIVL</td>
<td>A HEVSEISVRT VSPPEEETGE</td>
<td>9</td>
</tr>
<tr>
<td>HGPMIVL</td>
<td>A HEVSEISVRT VSPPEEETGE R</td>
<td>8</td>
</tr>
<tr>
<td>HGPMIVL</td>
<td>A HEVSEISVRT VSPPEEETGE R</td>
<td>8</td>
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<tr>
<td>HGPMIVL</td>
<td>A HEVSEISVRT VSPPEEETGE R</td>
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<tr>
<td>HGPMIVL</td>
<td>A HEVSEISVRT VSPPEEETGE</td>
<td>8</td>
</tr>
</tbody>
</table>

Sequences for HGPMIV and MiVI were deduced from nucleotide sequences; all others were determined by protein sequencing. The sequence shown for HGPMIVII and MiVIII is the only region experimentally determined; the sequence HGPMIV is not known. Polypeptides are aligned for maximum homology, and in each case only a portion of the extracellular domain is shown. In HGPA, numbers indicate residues. In HGPB, the lower case letters indicate residues not expressed but encoded by the corresponding sequence of the pseudoexon (see text). Note a gap in that pseudoexon sequence corresponding to residue 37 (Thr) of HGPA. In HGPMIII and MiVII that sequence is expressed and therefore the numbering of residues downstream from the gap should be one residue less than in HGPA. The underlined residues are stretches of "new" common sequences present in variant glycophorins but absent in parent glycophorins; they are homologous to the synthetic peptides, as indicated. (1) Residues of hybrid junctions; two possible sites of δ-α junction are shown in MiV.

*Adapted with permission*; (+) and (−) indicate positive or negative reactions of RBCs with the respective antisera. HGPMIVL is included in the Mi complex based on its structural similarity to HGPMV. *S* indicates examples of anti-S sera that react with Jl erythrocytes; reactions with other antisera tested are also indicated.

**MATERIALS AND METHODS**

**Peptide synthesis** Peptides were synthesized by the Laboratory for Macromolecular Analysis at the Albert Einstein College of Medicine, using a solid phase peptide synthesizer (ABI 430 A; Applied Biosystems, Foster City, CA) and standard programs. Peptides were desalted on Sephadex G-10 equilibrated with 10% acetic acid, and further purified by reverse-phase high-performance liquid chromatography (HPLC) on an Aquapore C18 reverse-phase column (4.5 × 250 mm; Brownlee, Foster City, CA) with a C4 guard column (32 × 15 mm; Brownlee) using a linear gradient of 10% to 50% acetonitrile (HPLC grade; Fisher, Springfield, NJ) for elution. The composition of the major peaks was verified by amino acid analysis as described. This analysis also provided a measure of the quantity of the peptides. The peptides were designated "Hi1", "Sh", "Hop", and "Mur" to reflect common serologic reactions of corresponding antisera with erythrocytes whose antigens shared a sequence homologous to each peptide (Table 1).

An aliquot of each peptide was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/mL and used directly for inhibition studies.

**Erythrocytes and antisera.** Erythrocytes of the Mi series were obtained from the frozen collection of Los Angeles Orange Counties Red Cross Blood Services and from RBCs provided by serum and RBC exchange programs or from commercial sources (Immunocor, Norcross, GA). MiV, MiL, and MiVIII RBCs were obtained from the following individuals: MiV RBCs from the son of J-1, the MiVIII RBCs from BHG, the MiL RBCs from M. Moulds (Houston, TX), and MiL RBCs were obtained from J-L.

The antisera were obtained from the in house collection of the Los Angeles Orange Counties Red Cross Blood Services or from commercial sources (Immunocor, Norcross, GA). Anti-Mur + Hut + Vw* were gifts from M. Moulds (Houston, TX). Anti-S designates examples of anti-S that agglutinated J-L. cells.

**Inhibition assays.** Before testing, the peptides were diluted in PBS to a concentration of 0.2 mg/mL. An equal volume of the diluted peptide and the antiserum were incubated for 15 minutes at room temperature. This process represented the inhibition portion of the test. Two controls were tested with each reagent in parallel with the peptide and antibody mixtures, a peptide control (peptide diluted with 6% bovine serum albumin [BSA]) and an antibody
Fig 1. Schematic representation of variant glycophorin genes and proteins. (The gene and the corresponding protein are on the same line.) L, leader sequence; Ex, extracellular domain; TM, transmembrane domain; Cyt, cytoplasmic domain; 3'UT, 3' untranslated region; ψ, pseudoexon; M/N, S/s, sites of blood group epitopes; 1 through 7 refer to exons in 5' to 3' direction; [J] a gene exons or corresponding expressed sequences; (II) δ gene exons or corresponding expressed sequences; (III) ψ gene pseudoexon or corresponding expressed sequences. The arrows indicate the junction sites of parent α and δ sequences within respective genes. Note that a portion of δ pseudoexon is expressed and adjoins a portion of α exon 3 in HGP MiIII and MiVI (see Huang and Blumenfeld for details); the vertical brackets show the location of synthetic peptides; in MiJ.L, the peptide is S<sup>ψ</sup>; in MiV the peptide is Hil; in MiIII the peptides are (from left to right) Mur and Hil; in MiVI the peptides are (from left to right) Mur, Hop, and Hil (see Table 1).

control (antibody diluted with 6% BSA). The peptide/antibody and control mixtures were then tested with the appropriate antigen-positive and antigen-negative RBCs. The results were considered valid when the peptide control was negative and the diluted antibody control was positive.

Due to limited quantities of reagents, tests using anti-Hop, anti-Hil, and anti-Mur were performed using a microcapillary technique as described by Myers and Reynolds.

Routine serologic techniques. Routine serologic techniques were used for testing control antisera for M, N, S, s, and S<sup>ψ</sup>. The mixtures with anti-M and anti-N were incubated at room temperature for 15 minutes. They were then centrifuged at 1,000g for 30 minutes at room temperature, washed four times with PBS, and then treated with antiglobulin reagent. After centrifugation at 1,000g the tubes were read for agglutination.

Interpretation of test results. Inhibition of the antibody by the peptide was noted to have occurred when the mixtures of peptide plus antibody plus antigen-positive RBCs resulted in no agglutination, whereas the appropriate controls were agglutinated with the specific RBCs.

Lack of inhibition was indicated if agglutination occurred both in mixtures of peptide plus antibody plus antigen-positive RBCs and in appropriate control mixtures.

RESULTS

Structures of synthetic peptides. The peptides were synthesized to include residues of the hybrid junctions in glycophorins MiJ.L., MiV, MiIII, and MiVI<sup>8,12,13</sup> and those unique to the expressed pseudoexon sequence in MiIII and MiVI<sup>8,13</sup> (Table 1). Additional amino acid residues surrounding the deduced “minimum” epitopes were included to ensure solubility of the synthetic peptides in aqueous buffers and to allow for some particular secondary structure. For example, prediction of the secondary structures by the method of Garnier et al<sup>19</sup> showed that the putative anti-Hil epitope may have a high propensity to form an α-helix. Finally, the synthesized peptides were tested individually for inhibition of hemagglutination of the respective Mi RBCs by Hil, S<sup>ψ</sup>, Hop, and Mur antisera.

Sequences of the synthetic peptides are shown in Table 1 (underlined residues). The Hil peptide of 14 amino acids is homologous to the sequence of residues 54 to 67 of MiV glycophorin or the identical sequence found at residues 53 to 66 of MiIII and MiVI glycophorins (the note to Table 1 explains the difference in residue numbers). The S<sup>ψ</sup> peptide is defined by an identical region of MiJ.L. glycophorin and differs from the Hil peptide by a single amino acid at residue 61, where a methionine in MiJ.L. is substituted by a threonine in each of the other three glycophorins.<sup>8,12,13</sup> The Hop peptide consists of 10 residues defined by residues 44 to 53 of MiVI glycophorin<sup>13</sup> that are also identical to residues 45 to 54 of MiVIII glycophorin<sup>11</sup> (Table 1). The Mur peptide represents residues 32 to 44 of MiIII and MiVI glycophorins.<sup>8,13</sup>

Inhibition of agglutination. The Hil peptide was specific in its ability to neutralize anti-Hil, as shown by its inhibition of hemagglutination with Hil-positive RBCs from Mi categories III, V, and VI (Table 2). The peptide did not inhibit agglutination with anti-S<sup>ψ</sup>, -Hop, -Mur, -M, -N, -S, or -s.
The failure to inhibit anti-S\textsuperscript{M} is particularly noteworthy because the S\textsuperscript{M} and Hil structures differ by only one amino acid (Table 1). In contrast, the S\textsuperscript{N} peptide only inhibited agglutination with anti-S\textsuperscript{N} and by similar tests did not neutralize anti-Hil, -Hop, -Mur, -M, -N, -S, or -s (Table 2). The methionine to threonine substitution was clearly critical for recognition by these antisera.

The specificity of the Hop peptide was shown by inhibition of the agglutination of the anti-Hop antiserum with MiVIII cells (Table 2). The peptide did not inhibit anti-Hil, -S\textsuperscript{N}, -Mur, -M, -N, -S, or -s.

The Mur peptide was shown to inhibit agglutination by anti-Mur of MiIII and MiVI RBCs. The anti-Mur serum used also contained anti-Hut and -V\textsuperscript{W} (no pure anti-Mur serum was available to us) (Table 2). The specificity of inhibition was shown by the failure of the Mur peptide to inhibit the agglutination of MiIII RBCs, which are known to be agglutinated specifically by anti-Hut but not by anti-Mur (Table 2). Possible interference of anti-V\textsuperscript{W} was not examined, because within the Mi series only Mi I RBCs are V\textsuperscript{W}-, and those RBCs are Hil-, S\textsuperscript{N}-, Mur-, and Hop-.

Surprisingly, the synthetic Mur peptide did not inhibit the agglutination when tested against two different examples of MiIV cells. These cells do react with anti-Mur sera (Table 2). The Mur peptide did not inhibit anti-Hil, -S\textsuperscript{N}, -M, -N, -S, or -s.

**DISCUSSION**

We have shown here that synthetic peptides homologous to unique polypeptide segments of Mi classes of variant glycophorins specifically inhibit the agglutination of the RBCs bearing these antigenic sequences by their specific antisera. The results allow us to conclude that the \(\alpha\)-\(\delta\) junction in MiIII, MiV, and MiVI glycophorins encompasses the epitopes for anti-Hil, the \(\alpha\)-\(\delta\) junction in MiJ.L. encompasses the epitopes for anti-S\textsuperscript{N}, the \(\delta\)-\(\alpha\) junction in MiVI glycophorin provides the epitope for anti-Hop, and the expressed \(\delta\) pseudoexon sequence in MiIII and MiVI glycophorins outlines the epitope for anti-Mur.

The four peptides were selected for synthesis because they represent sequences that were found to be identical in variant glycophorins occurring in individuals whose erythrocytes were known to share a common Mi determinant. The critical amino acid residues for antigenicity within the epitope apparently include the very same residues that join the \(\alpha\)-like and \(\delta\)-like glycophorin sequences; nevertheless, the adjoining residues must also have a determinative function. This is evidenced in particular by the fact that Hil and S\textsuperscript{N} antisera have different serologic reactivities and their corresponding antigens are \(\alpha\)-\(\delta\) hybrids that are identical, except for a single residue two amino acids downstream from the glycine that marks the \(\alpha\)-\(\delta\) junction site in all the hybrid glycophorins that react with these antisera.

In the case of the Mur antiserum, a string of residues unique to the expressed pseudoexon sequence is probably the critical epitope. Significantly, the Mur synthetic peptide inhibited hemagglutination by appropriate antisera when tested with MiIII and MiVI but not with MiIV RBCs; yet these three Mi-class RBCs all react with Mur antisera (Table 1). No information is available so far concerning the structure of MiIV glycophorin. Nevertheless, because MiIV RBCs react with anti-Mur, it is likely that this protein contains a portion of the expressed pseudoexon. At this time one can only speculate why the Mur synthetic peptide did not inhibit the hemagglutination of MiIV RBCs by anti-Mur; thus, either the junction of the expressed pseudoexon and the \(\alpha\)-glycophrin sequence in MiIV glyco-
phorin leads to a subtle structural variation that is not evident in the Mur peptide alone, or the serologic classification of the MiV erythrocytes could be more complex than previously suggested. In addition, the specificity of the antisera may not be absolute and this antisera may in fact contain yet another antibody reacting with MiIV RBCs. Of interest is that MiIV RBCs react identically to MiIII and MiVI RBCs when tested with a monoclonal antibody whose epitope may span a stretch of residues homologous to the expressed pseudoexon sequence.21

We had predicted that the Hop synthetic peptide would inhibit hemagglutination of both the MiVI and MiVIII RBCs with anti-Hop. Indeed, the Hop peptide inhibited the reaction of MiVIII cells, but no proper reagents were available to us for testing the MiVI cells. MiVI glycophorin is a δ-α-δ hybrid and the middle region of the Hop synthetic peptide (Thr-Thr-Val-Tyr) delineates its δ-α junction (Table 1). At the corresponding site, the sequence of MiVIII glycophorin is identical to MiVI. The sequence at that site in MiIII, MiV, MiL, or α-glycophorins, none of which react with anti-Hop, is Arg-Thr-Val-Tyr (Table 1); it would therefore appear that the Hop antisera recognized the Arg → Thr substitution. Significantly, the MiVII cells that bear the same substitution but show an additional change of Tyr21 → Ser21 do not react with the anti-Hop serum.20 Clearly, that substitution may change the specificity of the epitope.

In an approach similar to that used in this study, synthetic peptides and glycopeptides were used to determine the specificity of anti-Mi22 and anti-M and -N antibodies.23 The studies presented here show that peptides can be designed to investigate the relationship of structure to the immunoserologic behavior of the variant phenotypes such as those of the Mi complex. Peptides of the kind described here may make it possible to generate more specific antibodies that may help clarify the complexities of certain Mi-like phenotypes. Antibodies of that kind could also expand the range of antibody reagents available for a more precise classification of the Mi series of antigens.24 Inhibition by one of these defined peptides of a hemagglutination reaction of a specific antisera may provide information about the structural variations that resulted in the variant antigen. For example, we can now predict that glycophorins of all RBCs that are agglutinated by the anti-Hil serum would contain an α-δ junction.

The epitopes of other variant phenotypes of the MNSs blood group system, eg, St* or Dantu, are also contained in hybrid glycophorins.24-26 Application of an approach similar to that used here could establish whether the δ-α junction sequences of such hybrids are or contain the epitopes for the specific antibodies that recognize these variant antigens.

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