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Structural analysis of the Ss sialoglycoprotein specific for Henshaw blood group from human erythrocyte membranes

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The N-terminal structures of the MN and Ss erythrocyte membrane sialoglycoproteins (glycophorins A, B) from two Henshaw (He) blood-group heterozygotes were determined by manual sequencing of tryptic glycopeptides and various secondary fragments. No structural alteration of the MN glycoprotein could be detected. The He-specific portion of the Ss glycoprotein was found to exhibit the N-terminal sequence Trp-Ser-Thr-Ser-Gly- (+ = glycosylation). Thus it differs at three positions from its normal counterpart which possesses 'N' activity and exhibits the N-terminal structure Leu-Ser-Thr-Thr-Glu-. Analysis of the Ss glycoprotein from 15 He-negative erythrocyte samples did not reveal any of the three He-specific structural alterations. The presence of a glycine residue at the fifth position of the blood-group-M-active MN glycoprotein as well as in the He-specific Ss glycoprotein provides an explanation for the occurence of antisera (anti-M^e) reacting with the M and He antigens.

Previous studies have provided evidence that the human *MN* and *Ss* blood-group gene loci encode the amino acid sequences of two (MN and Ss) sialoglycoproteins (SGPs) (synonyms: glycophorins A, B) in red blood cell membranes [1–4]. Amino acid polymorphisms at the first (serine/leucine) and fifth (glycine/glutamic acid) positions of the major (MN) SGP determine MN blood-group specificity [5–10], whereas the Ss antigens appear to be dependent on a methionine/threonine polymorphism at position 29 of the Ss SGP [11–13]. The N-terminal 26 residues of the Ss SGP are identical with those of the blood-group-N-active major glycoprotein [9–12]. Therefore, the former molecule carries an additional N receptor, denoted as 'N' [1,3].

The Henshaw (He) erythrocyte antigen, which occurs in about 5% of blacks and only rather rarely in caucasians, is genetically associated with the MNSs receptors [1,14–17]. Serological studies have revealed that certain rabbit or human antisera, denoted as anti-M^e, react with the He and M antigens, suggesting that both receptors exhibit a structural similarity. In the present communication we describe studies on the SGPs from two *He* heterozygotes. Our data demonstrate that the He receptor represents an allelomorphic form of the 'N' antigen on the Ss SGP.

Abbreviations. BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; dansyl-, 1-(dimethylamino)naphthalene-5-sulphonyl-; DABITC, 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyanate; DABTH, 4-*N*,*N*-dimethylaminoazobenzene-4′-thiohydantoin; PITC, phenylisothiocyanate; SGP, sialoglycoprotein. Peptides are designated according to the SGP (MN or Ss) from which they were derived by trypsin (T), chymotrypsin (C), V8 protease (V) (after desialylation (d)) or cyanogen bromide (B) treatment, the elution during gel filtration (1 etc.) and the MN or Ss blood type of the red cells.

Enzymes. Neuraminidase, acylneuraminyl hydrolase (EC 3.2.1.18); trypsin (EC 3.4.21.4); chymotrypsin (EC 3.4.21.1); Staphylococcal V8 protease (EC 3.4.21.19).

MATERIALS AND METHODS

Blood units from two unrelated, *He* heterozygous black donors (genotypes *MS/NS* or *NS/Ns*) were drawn in Ann Arbor and Houston. It could not be elucidated with which *MNSs* haplotype *He* is associated. Blood samples from He-negative individuals were gifts of P. D. Issitt (South Florida Blood Service, Miami), P. Harris (American Dade Corporation, Miami) and S. Wilkinson (P. Hoxworth Blood Center, University of Cincinnati) or obtained in Houston and Cologne. Anti-He was detected in the serum of a blood donor at Gamma Biological Inc. (Houston). BNPS-skatole was purchased from Pierce Chem. Corp. (Rockford, USA). Other materials have been described in previous publications [3,7–9,11,12,18–22].

Analytical procedures

Sialic acid, protein and sodium dodecylsulfate/polyacrylamide gel electrophoretic analyses were performed by the methods of Warren [23], Lowry et al. [24] and Laemmli [22,25], respectively. Amino acids and amino sugars were determined on automated amino acid analyzers [18,20]. Data on amino acids given in tables is uncorrected; values of the amino sugars were corrected for destruction during hydrolysis, using *N*-acetylaminosugars as standards. Tryptophan was measured spectrofluorometrically [10]. Manual sequence analyses of peptides were performed by the DABITC/PITC double-coupling method [12,26,27] and dansyl-techniques [28,29]. Conversion of the thiazolinones to DABTH-derivatives was carried out in aqueous trifluoroacetic acid (2:1; v/v) at 52 °C for 50 min. Direct identification of glycosylated

DABTH-serine or DABTH-threonine at certain positions of peptides was performed by analyses of the aqueous phases after butyl-acetate extraction, as described previously [20]. For haemagglutination assays, a 5% suspension of red cells (20 µl) was incubated with an equal volume of serial twofold dilutions of serum prepared with saline (0.145 M) solution. Results were read macroscopically after 30 min at room temperature and centrifugation in a Sorvall CW-1 centrifuge. For inhibition tests, agglutinin (20 µl; titre 1:4) was incubated for one hour at room temperature with 20 µl of serial twofold dilutions of the inhibitor solution (10 mg/ml), before the erythrocyte suspension (20 µl) was added.

Preparation of red cells, membranes, SGPs and peptides

Red cell membranes were extracted by the phenol/saline method [19] and the SGP mixture was fractionated into the major (MN) glycoprotein and the mixture of minor SGPs by gel filtration in the presence of detergent [7–9]. N-terminal glycopeptides of the MN and Ss SGPs were purified by gel and DEAE-cellulose chromatography after tryptic digestion of the SGP fractions [10–12,30]. Secondary chymotryptic and V8 protease peptides were prepared after desialylation by mild acid hydrolysis and separated by gel filtration [12,20,30]. The mixture (T1/2) of the two N-terminal peptides NNT1 (residues 1–39) and NNT2 (residues 1–31) from the MN SGP [10,30] of the He+*NNSs* donor and the mixture of the secondary V8 protease fragments (fraction SsT1dV1; residues 1–28 and 6–28) from the Ss SGP of the He+*MNSS* donor, after reduction, were cleaved with cyanogen bromide for 7 h and 24 h, respectively [11,12,30]. Treatment of intact erythrocytes with neuraminidase, trypsin or α-chymotrypsin was performed as described elsewhere [3,19]. *N*-Acetylation and desialylation (mild acid hydrolysis) of SGPs were carried out as in a previous paper [20]. For BNPS-skatole treatment [31], 5 mg of SGPs were incubated with 10 mg of the reagent in 1 ml of 80% (v/v) acetic acid for 5 h at room temperature. Subsequently, the sample was reduced with thioglycolic acid [31]. All modified SGPs were recovered by gel filtration on a column of Sephadex G-25 [31] and lyophilization.

RESULTS

Sialic acid, gel electrophoretic and serological analyses

The sialic acid levels of membranes from the two He-positive red cell samples (102% and 96% for the *MS/NS* and the *NS/Ns* donor, respectively) were not significantly different from those of control ghosts possessing identical Ss blood types (SS membranes, 100%±7%, $n=3$; Ss membranes, 100%±6%, $n=3$) [4,8]. Sodium dodecylsulfate/polyacrylamide gel electrophoresis of the He-erythrocyte membranes, followed by staining for protein or carbohydrate, revealed normal patterns. The periodic acid/Schiff-staining intensities of the SGP bands were not significantly different from those of control ghosts run simultaneously (data not shown).

Treatment of intact erythrocytes with α-chymotrypsin removes the N-terminal, glycosylated domain of the Ss SGP, whereas trypsin has no significant effect on this molecule. The N-terminal portion of the MN SGP is partially or completely released by chymotrypsin or trypsin, respectively [3,30]. The reaction of anti-He was slightly enhanced after trypsin treatment and weakened or abolished after α-chymotrypsin or neuraminidase digestion, respectively, of intact red cells (Table 1). This suggests that the He-receptor is located on the glycosylated portion of the Ss SGP.

Table 1. *Agglutination of enzyme-treated He+MNSS red cells by anti-He*
∅=no agglutination by undiluted serum

Agglutination titre with treatment by			
None	trypsin	α-chymotrypsin	neuraminidase
1:8	1:16	1:2	∅

Table 2. *Inhibition of anti-He by SGP fractions and fragments from He+MNSS red cells*
The unfractionated SGP mixture treated by acetylation, mild acid hydrolysis or BNPS-skatole was inactive at 10 mg/ml

Inhibitor	Inhibitory activity
	mg/ml
Unfractionated SGP mixture	0.6
MN SGP	>10.0
Minor SGP mixture	0.15
MNT1/2 (residues 1–39/31)	>10.0
SsT1 (residues 1–32)	0.3

Anti-He was found to be inhibited by the unfractionated SGP mixture from He-erythrocytes (Table 2), which contained the MN SGP, Ss SGP and D SGP (=glycophorin C) in a ratio of about 1.0:0.2:0.1, as judged from periodic acid/Schiff-staining after sodium dodecylsulfate/polyacrylamide gel electrophoresis. The antigen activity was enriched in the minor SGP fraction, prepared by gel filtration [9], which predominantly (ca. 65%) contained the Ss SGP, apart from other glycoproteins (MN SGP ca. 5%, D SGP ca. 20% and additional minor bands, see [9]), as revealed by electrophoretic analysis. The N-terminal peptide (SsT1, residues 1–32) of the Ss SGP, purified after tryptic digestion of the mixture of minor SGPs [10,11] also inhibited anti-He. Treatment with BNPS-skatole, desialylation and *N*-acetylation of SGPs destroyed their He-activity, suggesting that tryptophan, sialic acid(s) and amino group(s) are involved in the binding site of anti-He.

Structural analyses of glycopeptides

In view of the known antigenic relationship between the M and He antigens [16,17], the mixture (T1/2, residues 1–39/31) of the two N-terminal tryptic glycopeptides (T1, residues 1–39; T2, residues 1–31 [10,30]) were prepared from the major SGP of both He erythrocyte samples. T1/2 fragments from the He+*MNSS* individual were further digested by V8 protease after desialylation. Glycopeptides from the He+*NNSs* donor were cleaved with cyanogen bromide. Amino acid analyses of all these fragments (Table 3), performed in comparison to corresponding peptides from He-negative red cells (data not shown), did not reveal any alteration. In addition, sequence analyses (see legend to Table 3) did not provide any evidence for an amino acid exchange.

The N-terminal tryptic peptide (SsT1, residues 1–32), presumed to carry the He antigen, was also isolated from the red cells of both *He*-heterozygotes. The normal, 'N'-active Ss SGP exhibits glutamic acid as the fifth residue. Therefore, it can be cleaved by V8 protease at this position after desialylation [12]. Analyses on the SsT1 preparations from the two *He*-heterozygotes (see below) revealed a glutamic acid and a glycine residue at the fifth position and two additional amino acid exchanges at the first and fourth positions. Therefore,

Table 3. *Amino acid compositions of peptides MNT1/2 and NNT1/2 from He erythrocytes and secondary V8 protease or cyanogen-bromide fragments*

Values are expressed as residues per peptide molecule. A dash denotes less than 0.1 residue. Sequence analyses by the DABITC/PITC method (10, 16, 4, 14 and 7 degradation cycles for the peptides T1/2, NNT1/2dV1, MNT1/2dV2, MNT1/2B1 and NNT1/2B2, respectively) did not provide any evidence for an amino acid substitution. $GalNH_2$ galactosamine; $GlcNH_2$, glucosamine, Hse, homoserine

Amino acid	Peptide and position					
	MNT1/2 1–39/31	MNT1/2dV1 1/6–39/31	MNT1/2dV2 1–5	NNT1/2 1–39/31	NNT1/2B1 9–39/31	NNT1/2B2 1–8
Asp	2.6	2.6	–	2.6	2.4	–
Thr	7.7	6.8	2.0	8.1	5.9	2.3
Ser	7.9	7.0	0.9	7.9	6.7	1.0
Glu	1.6	1.2	1.1	2.3	1.3	0.9
Pro	0.4	0.3	–	0.5	0.4	–
Gly	0.6	0.6	–	0.1	–	–
Ala	1.9	1.7	0.1	1.9	0.9	1.0
Val	2.1	2.0	–	2.0	1.2	1.0
Met	1.1	1.0	–	0.9	–	–
Ile	1.0	1.0	–	1.0	1.0	–
Leu	0.6	–	0.9	1.0	0.1	1.0
Tyr	1.1	1.1	–	1.4	1.2	–
His	2.1	1.8	–	2.0	1.9	–
Lys	2.1	1.9	–	2.1	2.0	–
Arg	1.3	1.2	0.1	1.3	1.1	–
Hse	–	–	–	–	–	0.9
$GalNH_2$	11.5	10.6	3.0	11.6	8.7	3.0
$GlcNH_2$	5.9	5.5	0.2	6.3	5.7	0.3

desialylated SsT1 (residues 1–32) from both donors was digested with V8 protease, in order to selectively release a N-terminal pentapeptide (HeSsT1dV2, residue 1–5) from the normal, 'N'-active peptide portion. Subsequent gel filtration yielded one fraction (HeSsT1dV1, residues 1/6–28) containing a mixture of two fragments (He-specific portion, residues 1–28; normal portion, residues 6–28). Analysis of fraction HeSsT1dV1 (residues 1/6–28) established that all three amino acid exchanges (see below) occur in one (the He-specific) portion of the peptides from the He-heterozygotes. Peptide fraction HeSsT1V1 (residues 1/6–28) from the He + *MNSS* donor was further cleaved with cyanogen bromide, in order to obtain a small fragment (HeSsT1dV1B2, residues 1–8) from the N-terminal end of the He-specific portion of SsT1 (residues 1–32). Desialylated SsT1 from that individual was also digested with chymotrypsin, in order to obtain additional fragments for sequencing. Amino acid analyses of all the fragments described above are shown in Table 4. As can be seen from the composition of SsT1 (residues 1–32), this fragment was predominantly or exclusively released by cleavage at residue 32, rather than at position 35 [12], probably due to chymotryptic activity in the trypsin preparation. As judged from the data in Table 4 and comparative analyses of glycopeptides from He-negative erythrocytes (data not shown), the He-specific SsT1 portion exhibits three amino acid substitutions within the N-terminal eight residues. No significant change in the degree of glycosylation was apparent from galactosamine analyses.

Sequencing data on the various fragments described above are summarized in Fig. 1. The He-specific portion of SsT1 (residues 1–32) was found to exhibit leucine→tryptophan, threonine→serine and glutamic acid→glycine substitutions at the first, fourth and fifth positions, respectively. As shown in Fig. 1, the threonine→serine exchange was not detected by the initial sequencing of SsT1. A glycosylated serine occurring together with a threonine is difficult to detect [12,26]. Furthermore, only a small amount of SsT1 was used for sequencing, in order to preserve material for the preparation of secondary peptides.

Previous sequence analyses of the N-terminal portion of the Ss SGP had been exclusively performed on proteins or peptides, pooled from several individuals [9–12]. Peptide SsT1 (residues 1–32/35) was therefore isolated from 15 He-negative donors (seven blacks and eight caucasians). The N-terminal five residues were investigated by DABITC/PITC sequencing; the fourth position was also determined by the dansyl-technique. None of the three He-characteristic amino acid substitutions could be detected.

DISCUSSION

Our serological data demonstrate that the He antigen is associated with the N-terminal glycopeptides (SsT1, residues 1–32) of the Ss glycoprotein, for which leucine→tryptophan, threonine→serine and glutamic acid→glycine exchanges were detected at the first, fourth and fifth positions, respectively. Destruction of He activity by chemical modification of SGPs suggests that the antigen is located in that region of the fragment. The He receptor is apparently coded for by an allele of '*N*', the specificity of which is determined by leucine and glutamic acid at the first and fifth positions, respectively, of the Ss SGP. Further evidence for this conclusion has been obtained by recent studies on an *individual* who possesses the rare genotype $MS^u/MsHe$ and whose red cells are devoid of 'N' antigen. An allo-anti-N made by this individual is directed against blood-group-N-active N-terminal octaglycopeptides [33].

Previous studies have shown that the receptors of many anti-M or anti-N sera are destroyed by the removal of sialic acid(s), attached to oligosaccharide(s) at the second, third

Table 4. *Amino acid compositions of peptides SST1 and SsT1 from He erythrocytes and secondary V8 protease, cyanogen-bromide or chymotryptic fragments*
For simplification the compositions of the V8 protease peptides from the He + *NNSs* donor are not shown. For further details see Table 3

Amino acid	Peptide and position									
	SsT1 1–32	SST1 1–32	SST1dC1 1–20	SST1dC2 21–32	SST1dV1 1/6–28	SST1dV2 1–5	SST1dV3 29–32	SST1dV1B1 9–28	SST1dV1B2 1–8	SST1dV1B3 6–8
Asp	1.2	1.1	0.1	1.1	1.2	0.2	–	1.1	0.1	–
Thr	5.7	5.4	4.2	0.9	4.3	2.0	–	4.2	0.8	0.1
Ser	8.0	8.2	6.1	1.9	7.5	1.1	–	7.3	1.8	0.2
Glu	3.5	3.5	0.6	3.1	2.0	1.2	1.2	2.2	0.1	0.1
Gly	2.5	2.6	0.6	2.1	1.6	0.1	1.1	1.1	0.9	0.3
Ala	1.1	1.1	1.0	0.1	1.0	0.1	–	0.1	1.0	1.0
Val	2.0	2.1	2.0	0.1	1.9	0.1	–	1.1	1.0	1.0
Met	1.5	1.9	0.9	0.9	0.9	–	1.0	–	–	–
Ile	1.0	1.0	–	1.0	1.0	–	–	1.0	–	–
Leu	1.5	1.6	0.6	1.0	0.1	1.0	1.0	–	–	–
Tyr	1.0	1.1	0.9	–	0.9	0.1	–	0.9	–	–
His	1.1	1.2	1.0	0.1	1.0	–	–	1.0	–	–
Lys	1.1	1.0	0.9	–	1.0	0.1	0.1	1.1	–	–
Arg	0.1	0.2	–	–	–	0.1	0.1	–	–	–
Trp	+	+	+	–	+	–	–	–	+	–
Hse	–	–	–	–	–	–	–	0.1	1.0	1.1
$GalNH_2$	11.8	11.1	8.6	2.4	8.6	2.8	0.1	8.0	2.5	0.2
$GlcNH_2$	0.2	–	–	–	–	–	–	–	–	–

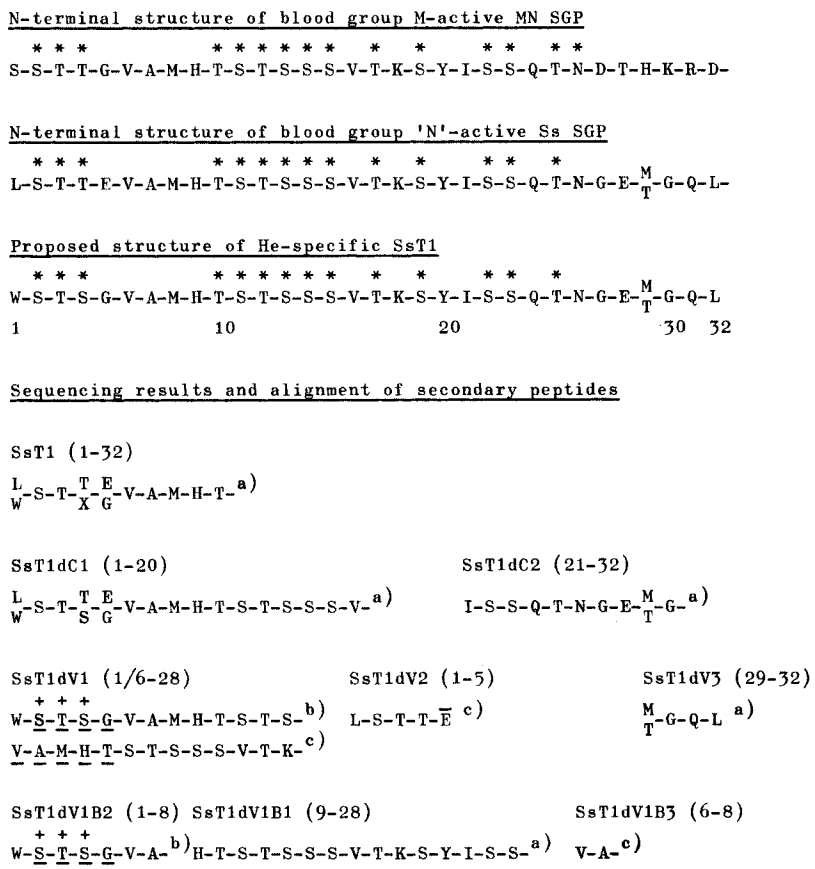

Fig. 1. *Amino acid sequence of He-specific SsT1, deduced from analyses of He-heterozygotes.* The N-terminal structures of the blood-group-M-active MN SGP and the normal, blood-group-'N'-specific Ss SGP are shown for comparison. The one-letter code for amino acids has been used [32]. (a) = sequence representing the He-specific and 'N'-specific peptide portion, (b) = sequence corresponding to the He-specific peptide portion, (c) = sequence representing the 'N'-active peptide portion (not aligned below the proposed structure of the He-specific SsT1). * denotes a glycosylation site. Unless otherwise indicated, amino acids were identified as DABTH derivatives. _ = identification as DABTH and dansyl derivative, = identification as dansyl derivative, $^+$ = direct identification of a glycosylated residue by analysis of the aqueous phase after butylacetate extraction [20]

and/or fourth position(s) of the SGPs [1,6,9,21,34]. Sialic acid(s) might represent a direct part of these antigenic determinants or stabilize a particular conformation of the N-terminal region of the SGPs, necessary for the binding of antibodies. The anti-He, which we have employed, is comparable to sialic acid-dependent anti-M or anti-N sera, since it did not react with desialylated red cells or SGPs. Samples of anti-He, which do not require sialic acid(s), have also been described [34].

The N-terminal tryptophan is apparently important for He activity, as measured with the single sample of anti-He which we have used. However, the significance of the serine and glycine residues at the fourth and fifth positions, respectively, is not clear. Given the polyclonal nature of antibodies, anti-He sera might require only one or two or all three of the He-specific amino acid exchanges. Since no structural alteration could be detected for the N-terminal region of the MN SGP from He-erythrocytes, the presence of a glycine residue at the fifth position of the blood group M-specific major SGP as well as in the He-active Ss SGP may account for the occurrence of antibodies (anti-M^e) reacting with both antigens [16,17]. Apparently, such antibodies do not distinguish between serine or tryptophan and threonine or serine at the positions one and four, respectively.

The two amino acid polymorphisms which represent the structural difference between MN antigens on the major SGP can be attributed to a minimum of two DNA base exchanges. Evidence has been obtained that the evolutionary development of the N antigen from the M receptor occurred via a rare gene encoding the M^c-specific amino acid sequence [35,36]. Similarly, the three He-characteristic structural alterations described above might be attributed to three independent amino acid and DNA base exchanges which had occurred sequentially during evolution. If this were so, two intermediate amino acid sequences should occur. In view of the uncertainty about the specificity of anti-He, discussed above, and the limited number of He-positive and He-negative erythrocytes which we have investigated by structural analyses, it is at present impossible to decide whether or not such intermediate amino acid sequences exist. When one of the possible mRNA sequences (UUG-UCU-ACA-ACU-GAG) coding for the N-terminal five residues of the N-active Ss SGP is read in the reversed direction, the sequence Ser^2-Thr^3-Thr^4 can be converted into Ser^2-Thr^3-Ser^4. The change from Leu^1 and Glu^5 into Gly^5 and Trp^1, respectively, requires three additional base exchanges (mRNA sequence UGG-UCA-ACA-UCU-GGU). Therefore, it is also conceivable that the He-specific structure evolved from an inversion of this gene segment. Development of the He-active Ss SGP by an inversion plus one DNA base exchange and unequal cross over of a gene encoding a M-specific MN SGP would also be possible.

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