Molecular Basis of the Globoside-Deficient Pk Blood Group Phenotype

IDENTIFICATION OF FOUR INACTIVATING MUTATIONS IN THE UDP-N-ACETYLGLACTOSAMINE:GLOBOTRIAOSYLKERAMIDE 3-β-N-ACETYLGALACTOSAMINYLTRANSFERASE GENE*

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* This work was supported in part by the Swedish Research Council, the Medical Faculty at Lund University, the Lund University Hospital Donation Funds, the Claes Högmå SAGMAN-stipendium and Tore Nilson’s Fund for Medical Research.

Running title: Molecular basis of the Pk phenotype

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SUMMARY

The biochemistry and molecular genetics underlying the related carbohydrate blood group antigens P, Pk and LKE in the GLOB collection and P1 in the P blood group system are complex and not fully understood. Individuals with the rare but clinically important erythrocyte phenotypes P1\(^k\) and P2\(^k\) lack the capability to synthesize P antigen identified as globoside, the cellular receptor for Parvo-B19 virus and some P-fimbriated \textit{E. coli}. As in the ABO system naturally occurring antibodies, anti-P of IgM and IgG class with haemolytic and cytotoxic capacity, are formed.

In order to define the molecular basis of the P\(^k\) phenotype we analyzed the full coding region of a candidate gene reported in 1998 as a member in the 3-\(\beta\)-galactosyltransferase family but later shown to possess UDP-N-acetylgalactosamine:globotriaosylceramide 3-\(\beta\)-N-acetylgalactosaminylntransferase or globoside synthase activity.

Homozygosity for different nonsense mutations (202C>T and 538insA) resulting in premature stop codons was found in blood samples from two individuals of the P2\(^k\) phenotype. Two individuals with P1\(^k\) and P2\(^k\) phenotypes were homozygous for missense mutations causing amino acid substitutions (Glu266Ala or Gly271Arg) in a highly conserved region of the enzymatically active carboxyterminal domain in the transferase. We conclude that crucial mutations in the globoside synthase gene cause the P\(^k\) phenotype.

**Key words:** P\(^k\) phenotype, anti-P, globoside, Gb\(_4\)Cer synthase, glycosyltransferase, blood group, allele, genotype, erythrocyte.
INTRODUCTION

The P<sup>k</sup> blood group phenotype was recognised in 1959 by Matson et al. because of antibodies in the serum of a patient whose erythrocytes were shown to lack an antigen related to but not identical with previously discovered P-related antigens (1). Further studies later showed the P antigen to be globotetraosylceramide (Gb<sub>4</sub>Cer, globoside, GalNAcβ3Galα4Galβ4GlcCer)<sup>1</sup>, the most abundant neutral glycolipid in the erythrocyte membrane (2) as well as in other mesodermally derived tissues (3,4). Later it was found that human fibroblasts with the P<sup>k</sup> phenotype were deficient in β-N-acetylgalactosaminyltransferase activity (5).

With only few exceptions the molecular basis of the major human blood group antigens have been determined and the genes responsible for erythroid expression of the various phenotypes cloned and characterized. However, the biochemistry and molecular genetics behind the carbohydrate-based P<sub>1</sub> antigen in the P blood group system (numerical nomenclature 003001 according to the International Society of Blood Transfusion, ISBT) and the P, P<sup>k</sup> and LKE antigens in the GLOB collection (ISBT no. 209001, 209002 and 209003) are complex and not yet fully understood (6).

Fig. 1 Figure 1 shows the biosynthetic route to these glycosphingolipid antigens by the sequential addition of monosaccharide residues to ceramide by different glycosyltransferases (7). The key enzyme for initiation of globo-series glycolipid synthesis, UDP-galactose:lactosylceramide 4-α-galactosyltransferase (Gb<sub>3</sub>Cer/P<sup>k</sup>/CD77 synthase, α4Gal-T), was independently cloned by three research teams in 2000 (8-10). Mutations in this gene were shown to constitute the molecular basis of the p phenotype in which all antigens of the P blood group system and GLOB collection are lacking on the cell surfaces (8,11). Attempts to find polymorphisms in the α4Gal-T gene correlating with P<sub>1</sub>+/P<sub>1</sub>- status were fruitless, however (8). Therefore, it is still unclear if another gene codes for P<sub>1</sub> synthase (also an α4Gal-T) or if other mechanisms exist.
Recently, the UDP-\(N\)-acetylgalactosamine:globotriaosylceramide 3-\(\beta\)-\(N\)-acetylgalactosaminyltransferase (Gb₄Cer/globoside synthase, EC2.4.1.79, \(\beta3\)GalNAc-T1) gene and its product was characterized by Okajima et al. using an expression cloning approach (12). Surprisingly enough, it turned out to be identical to a previously cloned gene, initially included in the 3-\(\beta\)-galactosyltransferase family based on genetic homology (13). Although it resembled other 3-\(\beta\)-galactosyltransferases no enzymatic activity could be defined in the original paper (13).

Cells of the p phenotype, which lack P1/P/P\(^k\) antigens, P\(_2\)^\(k\) phenotype which lack P1/P antigens and P\(_1\)^\(k\) phenotype which lack the P antigen are of principal interest because of potent naturally occurring antibodies which are regularly present in plasma. As in the ABO blood group system, antibodies of IgM and IgG class (anti-PP1P\(^k\), anti-P1P or anti-P) are made against the missing blood group antigens. Whilst the frequency of the p phenotype in general has been estimated at 5.8 per million (14) it is considerably higher in certain populations, especially in Northern Sweden (15). The frequency of the P\(^k\) phenotype is not known but it is generally accepted to be even rarer than p. The frequencies are possibly slightly higher in Finland and Japan but not as marked as for p in Northern Sweden (16). There are several reasons for the scientific and medical attention spent investigating patients with these rare phenotypes (17). P-related antibodies are implicated in haemolytic transfusion reactions if random antigen-positive blood is transfused. The P, P1 and P\(^k\) antigens are well developed on fetal erythrocytes but in most cases relatively mild haemolytic disease of the newborn due to the anti-PP1P\(^k\) or anti-P has been reported (16). Women of the p and P\(^k\) phenotypes suffer a higher frequency of spontaneous abortion than normal, a phenomenon that is most likely due to the IgG anti-P component (18). The placenta expresses high levels of P and P\(^k\) antigen (as opposed to the early fetus itself) and has been suggested as the prime target for these antibodies (19). Another anti-P-mediated disease usually seen in children...
following a viral infection is paroxysmal cold haemoglobinuria in which P positive erythrocytes are lysed by a transient auto-anti-P (20).

Globoside / P antigen has also been identified as a cellular receptor for parvo-B19 virus (21) that causes erythema infectiosum, also known as fifth disease, in children and sometimes is complicated by severe aplastic anaemia. Complete viral replication and subsequent cell lysis are limited to early erythroid precursor cells expressing the globoside receptor (22). Individuals lacking the receptor appear to be naturally resistant to this infection (23). Finally, it has also been shown that some P-fimbriated E. coli express globoside-binding molecules (PapF and PapG) at the tips of their pili (24), a finding with possible implications for their uropathogenicity.

Because of the relationship to many human pathogens and diseases the interest in glycolipid-based blood group antigens remains high. In this study we tested the hypothesis that the reported β3GalNAc-T1/globoside synthase (12) is indeed the enzyme responsible for synthesis of P blood group antigen. By analyzing the coding DNA sequence of the corresponding gene we show here for the first time that mutations capable of abolishing the enzyme’s function are present in individuals with the P^k phenotype.
EXPERIMENTAL PROCEDURES

**Blood samples.** Four blood samples of the P\(^k\) phenotype (one P\(_1^k\) and three P\(_2^k\)) were obtained from the liquid nitrogen in-house collection of test cells at the International Blood Group Reference Laboratory (IBGRL). The phenotype of the cells and presence of anti-P in serum were confirmed by standard serological methods at IBGRL and in two cases also by other reference laboratories. For screening of the detected missense mutations blood samples from apparently healthy random blood donors of mixed European descent were used.

**DNA preparation.** DNA was prepared from EDTA or acid-citrate-dextrose blood at the Blood Centre in Lund by a salting out method modified from Miller *et al.* (25) and dissolved in H\(_2\)O at a concentration of 100ng/\(\mu\)l.

Table 1  
**PCR amplification and DNA sequencing.** Oligonucleotide primers were synthesized by DNA Technology ApS (Aarhus, Denmark). For each reaction 5 pmol of primers P-(-6)-F and P-1015-R or P-123-F and P-891-R (Table 1) were mixed with 100 ng of genomic DNA, 2 nmol of each dNTP, 2% Glycerol, 1% Cresolred and 0.5 U AmpliTaq Gold (Perkin Elmer/Roche molecular systems, Branchburg, NJ) in the buffer supplied. The final reaction volume was 11 \(\mu\)l. Thermocycling was undertaken in a GeneAmp PCR system 2400 (Perkin Elmer Cetus, Norwalk, CT). Initial denaturation at 96\(^\circ\)C for 7 min was followed by 35 cycles at 94\(^\circ\)C for 30 s, 64\(^\circ\)C for 30 s, 72\(^\circ\)C for 1 min and a final extension for 2 min. PCR products were excised from 3\% agarose gels (Seakem, FMC Bioproducts, Rockland, ME) stained with ethidium bromide (0.56 mg/l gel, Sigma Chemicals, St. Louis, MO) and purified using Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany). The Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 Genetic Analyser (Applied Biosystems) were used for direct DNA sequencing according to the manufacturer’s instructions. Besides the PCR primers internal primers (Table 1) were used as sequencing primers. To avoid artefacts, sequencing was performed on both strands and using
independently obtained PCR fragments. Sequence analysis was performed with SeqEd software 1.03 (Applied Biosystems).

**Allele-specific primer PCR (PCR-SSP):** PCR with allele-specific primers designed to detect the two missense mutations found in the study was performed. For 797A>C detection 2.5 pmol of primer P-797C-null-F and P-1015-R were used together with 0.5 pmol of internal control primers JK-F3L and JK-R3L modified from (26) and PCR conditions as above. For 811G>A detection 7.5 pmol of primer P-811A-null-F and P-1015-R were used instead. For confirmation of homozygosity consensus primers P-797A-F and P-811G-F substituted P-797C-null-F and P-811A-null-F at 5 and 10 pmol per reaction, respectively. The annealing temperature for the latter reaction was increased to 65°C for optimal specificity.

**RESULTS**

**PCR amplification and DNA sequencing:** Using oligonucleotide primers shown in Table 1 the entire coding region of the β3GalNAc-T1 was amplified in one fragment in samples from donors with the phenotype P1k (n=1, English), P2k (n=3, Arabian, French, Finnish) or common GLOB collection/P blood group phenotypes (n=2, Swedish). Sequence analysis of the amplified gene fragments was performed and compared to sequences deposited in GenBank: AB050855 (12) and Y15062 (13). The Swedish random donors had β3GalNAc-T1 gene sequences identical to the consensus GenBank entries. However, in two of the P2k samples homozygosity for two distinct nonsense mutations resulting in premature stop codons were detected. The Finnish sample was homozygous for 202C>T resulting in an immediate stop codon following residue 67. The Arabian sample was homozygous for a single adenosine insertion at nucleotide (nt.) 537-538 (AG→AAG), here designated 538insA. This insertion causes a frame shift from amino acid 180 and a premature stop at codon 182 (consensus-Arg180-His181-Stop). The remaining two samples were homozygous for two
different missense mutations. In the English P1k sample glycine at residue 271 is substituted for arginine due to 811G>A whilst glutamic acid is changed for alanine at residue 266 due to 797A>C in the French P2k sample. DNA sequencing chromatograms visualizing the detected sequences from wild-type and Pk donors are found in Figure 2. A schematic representation of the open reading frames in the consensus and variant alleles of the β3GalNAc-T1 gene is shown in Figure 3.

Fig.2-4 A comparison of glycosyltransferase genes with significant homology revealed that both of the missense mutations found involve charged vs. non-charged residues in a highly conserved region of the carboxyterminal globular domain of the transferase (Figure 4). No other deviations from the β3GalNAc-T1 consensus sequence were encountered in any of the analyzed samples. No mRNA studies could be undertaken due to lack of fresh samples.

**Confirmation of homozygosity.** Since no other polymorphisms were detected in the β3GalNAc-T1 gene we ensured that both alleles were amplified for sequence analysis by using two independent PCR primer pairs, P-(-6)-F and P-1015-R or P-123-F and P-891-R. In two of the samples the presence of missense mutations and absence of consensus sequence were confirmed with genomic DNA and sequence-specific primers. Representative electrophoretograms are shown in Figure 5.

**Screening for missense mutations by sequence-specific primer PCR (PCR-SSP).** DNA samples of 220 mixed European blood donors were screened for the two missense mutations found during the study in order to exclude that they constitute common polymorphisms in the gene. Neither mutation was detected in any of the 440 alleles analyzed. Representative results outlining the screening procedure are shown in Figure 5.
DISCUSSION

The data presented in this paper show that mutations in the recently cloned β3GalNAc-T1 gene are associated with the clinically important Pk phenotype in which synthesis of the globoside/P antigen cannot take place. The four samples analysed were homozygous for rare alleles which is not unexpected considering the low frequency of this phenotype in all populations. This is most probably due to consanguineous marriages as previously shown in at least 7 of 33 studied propositi of the Pk phenotype (16). As is often the case with rare phenotypes when propositi from different ethnic groups and/or geographic areas are studied the molecular background for the samples in this study was quite heterogeneous. Similar data have been obtained for almost all rare blood group phenotypes with sporadic cases in most populations. Some of these variant phenotypes have a higher frequency in geographically isolated populations. A slightly increased frequency of the Pk phenotype has been noted in Japanese and Finns (16). It is therefore possible that the allele with 202C>T encountered in the Finnish sample is a founder gene responsible for the Pk phenotype also in other Finnish cases similar to the situation with the rare Jk3 negative blood group phenotype in Finns (27).

In two of the investigated samples nonsense mutations at nt. 202 or 538 truncate the protein to 20 and 55% of its native length, respectively, thereby making any retention of enzymic activity impossible since the enzymatically active domain is in the carboxyterminal portion. This agrees well with other blood-group-related glycosyltransferases that lose activity if severely truncated. For example, the common O allele (O') in the ABO blood group system has a single guanosine deletion at nt. 261 causing a reading frame shift that truncates any translated product at residue 117 (28) leaving no resemblance to A or B transferase after residue 86 (thus 25% of the amino acid sequence remains intact). Other examples are inactive forms of the FUT2 gene product, the 2-α-fucosyltransferase of the H blood group system, that are truncated at 55 and 61% of the full reading frame (29).
The other two P^k samples had missense mutations changing amino acids in the functionally active globular domain in the carboxyterminal portion of the transferase (30). By comparing the amino-acid sequence of homologous genes (Figure 4) it was shown that the mutated residues are either invariant or highly conserved among the sequences compared, suggesting their importance for the function of β3GalNAc-T. Furthermore, they are located in an evolutionarily conserved cluster of amino acids found in glycosyltransferases belonging to different functional families (β3Gal-T, β3GalNAc-T and β3GlcNAc-T) in humans and also in a Drosophila homologue, suggestive of importance for this region across species boundaries and enzyme specificities. Although not expressed and experimentally proven non-functional by enzyme analysis the data presented make it highly probable that both the Glu266Ala change from a large negatively charged to a small hydrophobic residue and the Gly271Arg change from a small non-charged to a large positively charged residue causes enough disturbance in this well-conserved region to render the protein inactive. Again, analogy can be taken from the ABO system where the full-length O^2 protein is rendered non-functional by a Gly268Arg change (31,32) at a conserved residue proposed to be exposed in the substrate-binding pocket of the 3-α-galactosyltransferase family (33).

Furthermore, it has been shown unambiguously (14) that the P1 antigen (putative gene located on chromosome 22) is inherited independently from the P antigen (globoside synthase gene located on chromosome 3). Thus, there is no reason to believe that Gly271Arg and Glu266Ala are specific for the P1^k and P2^k phenotypes, respectively. The frequency of the P1 antigen among Caucasians is approximately 75%. Any correlation of 811G>A (Gly271Arg) to P1+ status was effectively ruled out by PCR-ASP screening of the 220 samples, none of which were positive for the mutation whilst the majority was P1+. 
The P antigen may now be extracted from the GLOB collection and promoted to constitute a blood group system of its own according to the inclusion criteria set up by the ISBT working party on terminology for red cell surface antigens. The rule is that a blood group system has to be defined by a single genetic locus or possibly two (or three) closely linked loci so that the antigens included in that system are localized to the same (type of) molecule. Based on the data presented here the P antigen is now tied to a defined genetic locus different from other blood group loci and the molecular basis of the null (P1\textsuperscript{k} and P2\textsuperscript{k}) phenotypes of the system has been identified.

**Acknowledgements:** We thank Dr. Alan Chester at the Blood Centre, Lund, Sweden for carefully reading the manuscript and advice concerning carbohydrate and glycosyltransferase nomenclature.
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FOOTNOTES

1The abbreviations used are:

Gb4Cer, globotetraosylceramide, globoside, GalNAcβ3Galα4Galβ4GlcCer;
Gb3Cer, globotriaosylceramide, Galα4Galβ4GlcCer;
α4Gal-T, 4-α-galactosyltransferase, Gb3Cer/Pk/CD77 synthase;
β3GalNAc-T1, 3-β-N-acetylgalactosaminyltransferase, Gb4Cer/globoside synthase;
β3Gal-T1 to T5, 3-β-galactosyltransferase 1 to 5;
ISBT, International Society of Blood Transfusion;
PCR-SSP, polymerase chain reaction with sequence-specific primers;
nt., nucleotide;

Blood group antigens, phenotypes and antibodies are designated according to the nomenclature recommended by the ISBT working party on terminology for red cell surface antigens.

The nucleotide sequences for the variant globoside synthase (β3GalNAc-T1) genes from individuals of the blood group Pk phenotype have been deposited in the GenBank database under the following GenBank Accession Numbers: AF494103-6.

1. GenBank = GenBank Accession Number AF494103
2. GenBank = GenBank Accession Number AF494104
3. GenBank = GenBank Accession Number AF494105
4. GenBank = GenBank Accession Number AF494106
5. GenBank = GenBank Accession Number AB050855
6. GenBank = GenBank Accession Number Y15062
Table 1. Oligonucleotide primers used in this study.

| Primer name       | Nucleotide sequence (5’→3’)                         | Function |
|-------------------|-----------------------------------------------------|----------|
| P-(-6)-F          | GCTCGCGTGCTTCTGAGCTG                                | a, b     |
| P-123-F           | ACCTCAGCCTTCCCCCACTAC                               | a, b     |
| P-238-R           | GGAGGTCAACCAGAATGACCAG                              | b        |
| P-263-F           | CATTCTGGTGACCTCCCACC                                | b        |
| P-684-F           | AACCCATATTTCTACCAGGAG                               | b        |
| P-737-R           | TCCTTGGCACCAAATCTCTGG                               | b        |
| P-797C-F(null)    | ACGTAAAACCCATCAAGTTTGC                              | c        |
| P-797A-F          | CACGTAAAACCCATCAAGTTTGA                             | c        |
| P-811A-F(null)    | ATCAAGTTTGAAGATGTGTATGTCA                           | c        |
| P-811G-F          | TCAAGTTTGAAGATGTGTATGTCA                            | c        |
| P-891-R           | CGTCTCAGTTGACAGACATCC                               | a, b     |
| P-1015-R          | AAAGTATCCTGTCTCTTAGGC                               | a, b, c  |
| JK-F3L            | GCATGCTGCCATAGGATCATTGC                             | c        |
| JK-R3L            | GAGCCAGGAGGTTGTTGCC                                 | c        |

- a. primer used for amplification of PCR fragments for sequencing
- b. primer used as sequencing primer
- c. primer used for PCR-SSP to screen for presence of and confirm homozygosity for the two missense mutations
FIGURE LEGENDS

**Figure 1.** Biosynthetic pathway for globoside and related substances of the P blood group system and GLOB blood group collection.

**Figure 2.** DNA sequencing chromatograms showing the sequence surrounding the mutations found in each sample: 1, nt. 197-205 in the Finnish P₂^{k} sample; 2, nt. 535-543 in the Arabian P₂^{k} and 535-544 in the wild-type sample; 3, nt. 807-815 in the English P₁^{k} sample; 4, nt. 793-801 in the French P₂^{k} sample. In each quadrant the upper sequence shows the mutated sample and the lower is the consensus sequence from a wild-type sample. Arrows point at the mutated bases.

**Figure 3.** Schematic representation of the β3GalNAc-T1 coding sequence in wild-type and P^{k} phenotype donors. The bars show the open reading frames of the gene and the location of different mutations (depicted as thick vertical lines) found in this study. The upper bar represents the full coding sequence of consensus samples. The relative locations of primers used for amplification of DNA fragments for sequence analysis and mutation screening (indicated by footnotes a and c in Table 1) are shown as filled and open arrowheads above the bars, respectively. However, the primer targeted against the 3’-untranslated region of the gene was used for both purposes but is filled.

**Figure 4.** Multiple amino acid sequence alignment of homologous glycosyltransferases based on ClustalW plots presented in previously published papers (12, 13, 34, 35). Sequences from the enzymatically active globular domain of the carboxyterminal portion in the transferases corresponding to amino acids surrounding the residues mutated in two of the P^{k} samples are
shown. Numbering on the left and right refers to the first and last amino acid shown on each line, respectively. The sequences are from human β3GalNAc-T1 (12) originally designated β3Gal-T3 (13), human β3Gal-T1,2 and 4 (13), human β3Gal-T5 (34), human β3GlcNAc-T with poly-N-acetyllactosamine synthase activity also designated β3Gn-T (35), a homologous gene, GT9, that was initially published as β3Gn-T (35) and a Drosophila melanogaster gene homologue to the human β3Gal-T gene family designated Brainiac (36). The two upper lines show the sequences from the English and French Pk individuals. Hyphens represent sequence identity to β3GalNAc-T1. Light grey, dark grey and black boxes symbolize identity between 4-5, 6-7 or all 8 of the compared sequences, respectively.

**Figure 5.** Representative gel electrophoretograms showing PCR-SSP screening for missense mutations found in the globoside synthase gene of individuals with the Pk phenotype. PCR-SSP products were amplified with the primer combinations described (see Experimental procedure and Table 1) and separated on 3% agarose gels. The upper (nt. 797) and lower (nt. 811) panels show screening with primers amplifying the mutated (left) or consensus (right) sequence. Samples from individuals with the following phenotypes are shown:

Lane 1, Pk phenotype donor homozygous for the 797A>C (upper) or 811G>A (lower) mutations; lane 2, Pk phenotype donor negative for the mutation tested for; lanes 3-4, random donors of common phenotype; 5, H2O contamination control.

M is the molecular size marker φX174RF DNA HaeIII (Life Technologies, Gaithersburg, MD, USA). Filled and thin arrows indicate the globoside synthase-specific fragments (sizes given in basepairs) and the JK-blood-group-gene-derived control (952 bp) DNA fragments, respectively.
Figure 1.

- Lactosylceramide (LacCer)
  - 3-β-N-acetylglucosaminyltransferase
  - 4-α-galactosyltransferase
    - Lactotriaosylceramide
      - 4-β-galactosyltransferase
        - Paragloboside (type 2 precursor)
          - 4-α-galactosyltransferase
            - H, A, B transferase
              - P1 antigen
              - ABH antigen
          - 3-β-N-acetylgalactosaminyltransferase
            - Globoside (Gb₄Cer) P antigen
Figure 2.
Figure 3.
### Figure 4.

| Protein | Sequence |
|---------|----------|
| Pk (Engl.) | M M G H V K P I K F E D V Y V G I C L N L K V N I H I |
| Pk (Fr.) | T S L I H T R L L H L E D V Y V G I C L R K L G I R H F F Q |
| β3GalNAc-T1 | V S L G I R R L L H L E D V Y V G I C L A K L R I C D P L F E |
| β3Gal-T1 | V S L G I R R L L H L E D V Y V G I C L R K L G I R H F F Q |
| β3Gal-T2 | V S L G I R R L L H L E D V Y V G I C L A K L R I C D P L F E |
| β3Gal-T4 | V S L G I R R L L H L E D V Y V G I C L A K L R I C D P L F E |
| β3Gal-T5 | V S L G I R R L L H L E D V Y V G I C L A K L R I C D P L F E |
| GT9 | S R E Y L R A W H S E D V S L G T W L A P V D V Q R E H |
| β3GlcNAc | A T S R V H L Y P I D D V Y T G M C L Q K L G I V E P R E K |
| Brainiac | A S V H L P L F R F D D V Y L G I V A L K A G I S L Q H |
Figure 5.
Molecular basis of the globoside-deficient Pk blood group Phenotype. Identification of four inactivating mutations in the UDP-N-acetylgalactosamine:globotriaosylceramide 3-beta-N-acetylgalactosaminyltransferase gene

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J. Biol. Chem. published online May 21, 2002

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