Molecular Defects Underlying the Kell Null Phenotype*

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Expression of the Kell blood group system is dependent on two proteins, Kell and XK, that are linked by a single disulfide bond. Kell, a type II membrane glycoprotein, is a zinc endopeptidase, while XK, which has 10 transmembrane domains, is a putative membrane transporter. A rare phenotype termed Kell null (Ko) is characterized by the absence of Kell protein and Kell antigens from the red cell membrane and diminished amounts of XK protein. We determined the molecular basis of eight unrelated persons with Ko phenotypes by sequencing the coding and the intron-exon splice regions of KEL and, in some cases, analysis of mRNA transcripts and expression of mutants on the cell surface of transfected cells. Six subjects were homozygous: four with premature stop codons, one with a 5’ splice site mutation, G to A, in intron 1, and one with an amino acid substitution (S676N) in exon 18. Two Ko persons with premature stop codons had identical mutations in exon 4 (R128Stop), another had a different mutation in exon 4 (C838Stop), and the fourth had a stop codon in exon 9 (Q348Stop). Two Ko persons were heterozygous for two mutations. One had a 5’ splice site mutation (G to A) in intron 3 of one allele that caused aberrant splicing and exon skipping, and the other allele had an amino acid substitution in exon 10 (S363N). The other heterozygote had the same amino acid substitution in exon 10 (S363N) in one allele and a premature stop codon in exon 6 (R192Stop) in the other allele. The S363N and S676N mutants, expressed in 293T cells, were retained in a pre-Golgi compartment and were not transported to the cell surface, indicating that these mutations inhibit trafficking. We conclude that several different molecular defects cause the Kell null phenotype.

Two distinct proteins, Kell and XK, linked by a single disulfide bond, are responsible for expressing the Kell blood group antigens. A single antigen, Ks, resides on XK, a protein that spans the red cell membrane 10 times, and over 23 antigens are carried by Kell protein, a 93-kDa, type II membrane glycoprotein. This topic has been reviewed (1, 2). The molecular basis for most of the Kell antigens have been determined and are due to base mutations encoding single amino acid changes (3). In rare cases, red cells lack either XK or Kell protein. Red cells that lack XK have the McLeod phenotype, and red cells that lack Kell protein and Kell antigens have the Kell null (Ko) phenotype (1, 2).

McLeod red cells, in addition to lacking XK and its associated antigen Kx, have a greatly reduced amount of Kell protein and all of its antigens (4, 5). The function of XK is not known, but it has a structure that resembles a membrane transporter (6), and a homolog, Ceds-8, is involved in regulating programmed cell death in Caenorhabditis elegans (7). XK is expressed in many tissues, other than erythroid, primarily in skeletal muscle, brain, and pancreas (6). Although the function of XK is not known, its possible physiological role may be gleaned from a set of clinical symptoms that are associated with the McLeod phenotype, including red cell acanthocytosis and late onset forms of muscular and neurological disorders (8).

Unlike McLeod phenotype red cells, Ko red cells have normal shape. Ko red cells have a reduced amount of XK protein (9), but, parenthetically, they have enhanced Ks antigen activity (1, 2, 10). Kell is a zinc endopeptidase and a member of the M13 or nephrilysin family whose principal functions are the activation of bioactive peptides by proteolytic cleavage of larger inactive polypeptides (11). Included in this family are neutral endopeptidase 24.11, two endothelin-converting enzymes (ECE-1 and ECE-2), the product of the PEX gene, and XCE, which is preferentially expressed in the central nervous system (12, 13). Neutral endopeptidase 24.11 is a widely distributed and promiscuous enzyme with many substrates. Its multiple roles and specificities probably depend on cellular location and availability of substrates (14, 15). ECE-1, ECE-2, and Kell, on the other hand, have narrow substrate specificity. ECE-1 and ECE-2 primarily activate endothelin-1, although they also can act on big endothelin-2 and -3, producing endothelin-2 and endothelin-3 (16). ECE-2 has an acidic pH optimum and has an intracellular role (17). By contrast, Kell preferentially cleaves the inactive precursor, big endothelin-3, at Trp21-Ile22, producing a 21-amino acid vasoactive peptide, termed endothelin-3.

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The abbreviations used are: Ko, Kell null; ECE, endothelin-converting enzyme; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RFLP, restriction fragment length polymorphism; ER, endoplasmic reticulum; bp, base pair(s); CMV, cytomegalovirus; nt, nucleotide.

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Kell Null Genotypes

Kell also cleaves big endothelin-1 and -2 but to a much lesser extent than it does big endothelin-3 (11). An enzyme that primarily activates endothelin-2 has not yet been identified. Expression of Kell is not confined to erythroid tissues; there is near equal expression in testis and lower, but significant, amounts in many other tissues (18).

As a group, the endothelins play many different physiological roles. Primarily they function in the regulation of blood pressure by affecting contraction and proliferation of vascular smooth muscle, and they also have vasodilator effects via endothelin-mediated release of nitric oxide. The endothelins are also involved in mitogenesis and developmental processes by affecting the differentiation and migration of neural crest-derived cells (19–26). The role that Kell, as an endothelin-3-converting enzyme, plays in these processes and whether or not it has a complementary role with XK are not known.

Several McLeod phenotype results from different genetic defects. Several mutations abolishing or reducing XK mRNA splicing have been described, as have single base deletions and point mutations in the coding regions of KEL (6, 27–30). The molecular defects causing the Ko phenotype have not been extensively studied, and only a recent report on a single Ko person with a 5′ splice site defect has been described (31). We now report on eight unrelated Ko persons among whom we have identified several different molecular defects within the gene encoding Kell protein (KEL). These newly identified KEL mutations lead to alternative RNA splicing, premature stop codons, or amino acid substitutions that affect protein trafficking, all of which can cause the Ko phenotype.

**EXPERIMENTAL PROCEDURES**

**Serology**

Red cell typing was performed by the indirect antiglobulin test with well characterized antibodies and appropriate controls. The Ko phenotype was first identified at the various blood centers from which they originated and in most cases were confirmed at the Immunohematology Reference Laboratory of the New York Blood Center.

**DNA Preparation, Polymerase Chain Reaction, and DNA Sequencing**

Genomic DNA was prepared from the buffy coat of whole blood, and the coding regions of KEL were amplified by PCR, as previously described (32), with sets of primers that cover the entire open reading frame of the 19 exons, including the flanking intronic regions. The primers used for amplification of each exon are described in Table I. Some of the primers were described previously (33). The PCR products were separated by gel electrophoresis using 0.8% low melting agarose. The molecular defects causing the Ko phenotype have not been extensively studied, and only a recent report on a single Ko person with a 5′ splice site defect has been described (31). We now report on eight unrelated Ko persons among whom we have identified several different molecular defects within the gene encoding Kell protein (KEL). These newly identified KEL mutations lead to alternative RNA splicing, premature stop codons, or amino acid substitutions that affect protein trafficking, all of which can cause the Ko phenotype.

**Table I**

Sets of primers used to amplify KEL exons

| Exons | Primer name | Sequence |
|-------|-------------|----------|
| 1     | e1F         | 5′-AGATAAAGGGGGAGGAGAGGCCTG-3′ |
| 2     | e1R         | 5′-CTTACATCTCCCTCTCTCCCTAGTG-3′ |
| 3     | ei1F        | 5′-CCCCATGTTGGATAGCGCTTCA-3′ |
| 4     | ei1R        | 5′-GGGAGGAGCAGAGGTTAACAG-3′ |
| 5, 6  | ei5F3       | 5′-TTATGATATAGGGCATGCTCCCAAC-3′ |
| 7, 8  | ei7F        | 5′-CTATCCCCACCTGCCAACAATG-3′ |
| 9     | ei9F        | 5′-CTATCCAGTTGGCTTTCCTTCAT-3′ |
| 10    | ei10F       | 5′-TGCACCTTCACTGATGCTG-3′ |
| 11, 12 | ei10Ra   | 5′-TGCCCCGATATTCCCTCAAGAAG-3′ |
| 13    | ei12F       | 5′-CTTACCCTCCACAGGGAAG-3′ |
| 14    | ei13F       | 5′-TCTGTTCTTCTCCCTCCACACCT-3′ |
| 15    | ei14F       | 5′-CTTATGATATAGGGCATGCTCCCAAC-3′ |
| 16    | ei15R       | 5′-TTGTTCTTCCCTCCACACCT-3′ |
| 17, 18| ei17F       | 5′-GCTGATGAGGATCAGCAGAAGG-3′ |
| 19    | ei19R       | 5′-GCTGATGAGGATCAGCAGAAGG-3′ |

**Restriction Fragment Length Polymorphism of PCR Products**

C1162T Mutation—The C1162T mutation was confirmed by a restriction fragment length polymorphism (RFLP) analysis. A 310-bp DNA fragment that includes exon 9 was amplified from genomic DNA as described above, and the PCR products from common Kell-type DNA and from DNA containing the C1162T mutation were treated with TspI restriction enzyme (New England Biolabs, Beverly, MA). The C1162T mutation abolishes the TspI restriction site. The digested PCR products from common Kell type and the variant were separated by gel electrophoresis using 0.8% agarose.

G2147A Mutation—The G2147A mutation was confirmed by an RFLP analysis using Alu restriction enzyme. A 174-bp DNA fragment from KEL exon 18 of wild type and Ko DNA was amplified using the forward primer, in1F, described in Table II, and the following reverse primer: CTTGGGCGGGAGAGGCGCCGCTG. The PCR product was subjected to AluI digestion, and the digested PCR product was analyzed on 3.0% agarose gel electrophoresis. The G2147A mutation abolishes the AluI site.

G to A, 5′ Intron 3 Mutation—A 118-bp DNA fragment containing the 3′ portion of exon 3 and the 5′ splice junction of intron 3 was amplified from genomic DNA using the forward primer, 5′-CAGTGCCACG- GCCGGTGC, and the reverse primer, 5′-GGGAGGCGAGAGG-3′. PCR products were digested with NlaIII according to the manufacturer’s instructions (New England Biolabs) and analyzed by 3.0% agarose gel electrophoresis.

G1208A Mutation—A 120-bp DNA fragment encompassing exon 36 in exon 10 was amplified from genomic DNA using the forward primer, 5′-CCATAGGACCCTTCTCAGGAG-3′ and the reverse primer, 5′-TGTGCTTGCGTCTCC-3′. PCR products were digested with NlaIII according to the manufacturer’s instructions (New England Biolabs) and analyzed by 3.0% agarose gel electrophoresis.

Reverse Transcription of Seattle Ko RNA

RNA was prepared from blood of Seattle Ko using TRIZOL LS Reagent (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer.

The RNA was reverse transcribed and PCR-amplified using the PerkinElmer Life Sciences GeneAmp RNA PCR Kit and the company’s protocol (Applied Biosystems Division). The following PCR primers were used to amplify nt 54 (exon 1) to nt 823 (exon 7) of Ko cDNA: forward primer, CCCAGTGCCCGATCAGTCCCTA; reverse primer, TTGGGGGACATCAAAAATCTGCT.

The PCR products were either directly sequenced or subcloned in p-Adv vector (CLONTECH Laboratories, Inc., Palo Alto, CA) and sequenced in the Microchemistry Laboratory at New York Blood Center using a PerkinElmer Life Sciences model 373XL DNA Sequencer.
**TABLE II**

Primers used to sequence the 19 KEL exons

| Exon | Primer name | Sequence |
|------|-------------|----------|
| 1    | in0F        | 5'-AGCATAGAGGAGGCTACCACCCAGA-3' |
| 2    | in1R        | 5'-GGAGGAGGCTACAGCACCAGGG-3' |
| 3    | in2R        | 5'-GATGATGATGATTGTTGATGGGAT-3' |
| 4    | in3F        | 5'-TTTCTTCTCTCTCTCTCTCTCTC-3' |
| 5    | in4F        | 5'-TGAGAGGAGGAATGTCACCAGCC-3' |
| 6    | in5F        | 5'-GGATGGTTCCACCGACTGTTAGT-3' |
| 7    | in6R        | 5'-GATTGCTGATAGTGGGAGGAG-3' |
| 8    | in7F        | 5'-ACCTCCCCACACTGCTCCTC-3' |
| 9    | in8F        | 5'-CTCTCTCTCTCTCTCTCTCTC-3' |
| 10   | in9R        | 5'-GAGCTCTTCCTCATTCTCATTGTAC-3' |
| 11   | in10F       | 5'-GAGGAGGACAGGAGGAGGAGG-3' |
| 12   | in11F       | 5'-TTTCTTCTCTCCTCTCTCTCTC-3' |
| 13   | in12R       | 5'-TCCTGTTGCTGCTGCTGCTGCTG-3' |
| 14   | in13R       | 5'-TCCTGTTGCTGCTGCTGCTGCTG-3' |
| 15   | in14R       | 5'-TCCTGTTGCTGCTGCTGCTGCTG-3' |
| 16   | in15R       | 5'-TCCTGTTGCTGCTGCTGCTGCTG-3' |
| 17   | in16R       | 5'-ATGGTACCTAGAGAGGCTGAC-3' |
| 18   | in17R       | 5'-GAGCTTCAGTAATAAGGCGTTCAAC-3' |

**Expression Vectors with S363N or S676N mutations or with S650N as a Control**

**S363N**—A 500-bp segment of Kell cDNA between PpuMI (nt 753) and BstEII (nt 1230) restriction enzyme sites was amplified from Kell cDNA in order to create a G2108A mutation. The G2108A mutation was incorporated in the reverse primer, which contained a BstEII enzyme site. The following primers were used: forward primer, GGCACTTCCCTTTTTTGTCA; reverse primer, AGGTCTACACACACGAGCCAG. Primer 2316Ra, which contains underlined base introduces the point mutation. Primer 2316Rb, which contains underlined sequence shows the other underlined sequence shows the BstEII site. The PCR product was subcloned in pT7Blue vector (Novagen, Madison, WI), and the plasmid DNA containing the G2147A mutation was cut with Nhel and PmlI to release a 240-bp insert DNA. The 240-bp DNA segment was used to replace a corresponding segment in Kell cDNA present in pRc/CMV vector. The plasmid with the G2069A mutation was cut with Nhel and PmlI to release a 240-bp DNA segment. The 240-bp DNA segment replaced a similar 240-bp DNA segment in Kell cDNA present in pRc/CMV vector that had also been cut with Nhel and PmlI.

**S650N**—A PCR amplification was carried out in which the forward primer contained the G2069A mutation (S650N). The forward primer was GGCTGACCTATCGAGTGAGCTGACATAAC. The penultimate underlined base introduces the point mutation. Primer 2316Ra, which contains underlined sequence shows the other underlined sequence shows the BstEII enzyme site. The PCR product was subcloned in pT7Blue vector (Novagen, Madison, WI), and the plasmid DNA containing the G2147A mutation was cut with Nhel and PmlI to release a 240-bp DNA segment. The 240-bp DNA segment replaced a similar 240-bp DNA segment in Kell cDNA present in pRc/CMV vector that had also been cut with Nhel and PmlI.

**Protein Separation by SDS-PAGE and Western Immunoblotting**

Red cell membrane proteins (30 μg/lane), prepared as previously described (35), were separated on 4–12% SDS-polyacrylamide gels in reducing or nonreducing conditions, transferred to nitrocellulose membranes, and stained with 0.5% (w/v) Ponceau S (Eastem Kodak Co.) in 1% acetic acid (v/v). The membranes were treated with a rabbit polyclonal antibody to Kell protein (18), and the immune complex formed was detected using a second antibody conjugated with horseradish peroxidase. The horseradish peroxidase-conjugated antibiod was detected with a chemiluminescent substrate as previously described (35,18).

**Transfection of 293T Cells**

**Human embryonic kidney 293T cells (ATCC CRL 1573), grown in a minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum, were transfected using LipofectAMINE reagent (Invitrogen) according to the manufacturer’s recommendation.**

**Biotinylation and Isolation of Cell Surface-exposed Proteins**

Transfected 293T cells, grown to confluence on 100-mm plastic Petri dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ), were washed with PBS and then incubated for 30 min on ice with 3 ml of PBS containing 0.5 mmol/liter L-[35S] methionine (PerkinElmer Life Sciences). The lysate was then centrifuged at 2000 rpm for 1 min at 4 °C in a microcentrifuge. The pellets containing nuclei and unbroken cells were discarded, and the supernatant fractions were further centrifuged at 14,000 rpm for 15 min to obtain cell membranes. Membrane preparations were dissolved in SDS buffer (0.125 mol/liter Tris-HCl, pH 6.8, 1% SDS, 5% glycerol), separated on precast 4–12% Tris-glycine gel (Novex, San Diego, CA), transferred to nitrocellulose membranes, and stained with 0.5% (w/v) Ponceau S in 1% acetic acid (v/v). Equal loading and transfer of proteins to nitrocellulose was demonstrated by densitometry analysis of Ponceau-stained bands. The membranes were treated with a rabbit polyclonal antibody to Kell protein, and the immune complex formed was detected using a second antibody conjugated with horseradish peroxidase and a chemiluminescent substrate (Pierce).

**Metabolic Labeling of Transfected 293T Cells with L-[35S] Methionine**

Two days after transfection, 293T cells, grown to confluence, were washed twice with PBS and incubated at 37 °C for 20 min with 1 ml of L-[35S] methionine (PerkinElmer Life Sciences; specific activity 1110 Ci/mmol). Cells were then washed twice with PBS and “chase” incubated at 37 °C with growth medium containing 10
Endothelin-3-converting Enzyme Assay

Red cells were incubated with big endothelin-3 as substrate, and the amount of endothelin-3 produced was measured by enzyme immunoassay as previously described (11).

RESULTS

Brief Medical Histories—Michigan Ko was a 42-year-old African-American woman with a 4-year history of cardiomyopathy who was referred to a hospital for a heart transplant. Pretransplant tests identified the presence of an antibody in her serum compatible only with Ko red cells (anti-Ku).

North Carolina Ko was a 79-year-old African-American woman who entered the hospital for a left total knee arthroplasty due to osteoarthritis, at which time her antibody to Kell was detected. Secondary diagnoses included hypertension, cardiomegaly, depression, and gastroesophageal reflux.

The Seattle Ko index case was a 60-year-old white woman who was identified as having the Ko phenotype following a routine blood donation. She had no prior history of medical illnesses or blood transfusion, and her serum did not contain any red cell antibodies. The index case had three siblings (two sisters and one half-sister) and one living child, a 35-year-old daughter. One of the index case's full sisters also carries the Ko phenotype.

Portugal Ko was an obstetric patient who had received a blood transfusion after the birth of her first child. In preparation for the delivery of her second child, it was determined that her red cells had the Ko phenotype and her serum had an antibody to Kell (anti-Ku). No major medical problems were identified (37).

New York Ko was a middle-aged apparently healthy woman who, as a blood donor, was identified as having the Ko phenotype. She had not been transfused and did not exhibit antibodies in her serum.

Reunion Island contains a relatively large population of Ko individuals. Ten persons have been diagnosed with the Ko phenotype, and consanguinity is frequent. Detection of the Ko phenotype was made after some of the Ko persons received blood transfusions and developed antibodies to Kell; two were identified due to hemolytic disease of newborns, and others were identified by sibship screening (38).

Yugoslavia Ko, a 27-year-old pregnant woman with serum antibody to Kell red cell antigens, was detected in 1997 in a prenatal diagnostics laboratory.

Israel Ko, an Israeli Arab, is the brother of a known Ko patient who was admitted to a hospital in her 33rd week of pregnancy with vaginal bleeding. Israel Ko and a second brother with the Ko phenotype were identified by screening the immediate family.

Single Base Mutations Causing Premature Stop Codons—DNA sequencing of all 19 KEL exons and the flanking splice site regions showed that four unrelated Ko persons had single, homozygous mutations causing premature stop codons. The sequencing data are summarized in Table III.

Two of the Ko individuals (Michigan and North Carolina) had identical (C502T) mutations in exon 4. The C502T mutation changes a CGA codon encoding arginine, at position 128, to TGA, a stop codon. DNA sequencing also demonstrated that these two unrelated Ko individuals, both African-Americans, had the KEL6 genotype (T1910C, L597P).

One of the Ko individuals (Yugoslavia) also had a single base mutation in exon 4, but in a different location (T366A). The mutation is homozygous and converts a TGT codon encoding cysteine at position 83 to a TGA stop codon.

The fourth Ko person (Portugal) with a premature stop codon had a homozygous single base mutation, C1162T, in exon 9. The mutation converts a CAA codon encoding glutamine, at position 348, to a TAA stop codon. Exon 9 of the mother, father, and two children was also sequenced and contained a heterozygous C1162T mutation. These mutations were confirmed by RFLP analysis. A 310-bp DNA sequence from exon 9 that includes the site of mutation was amplified by PCR from genomic DNA and treated with a restriction enzyme. The C1162T mutation abolishes a Tsp45I site; thus, DNA without the mutation yields two fragments of 220 and 90 bp, while that with the mutation remains uncut. A heterozygous PCR product, treated with Tsp45I, would produce three bands, the uncut 310-bp fragment from one allele and the 220- and 90-bp fragments from the other allele. As shown in Fig. 1, Portugal Ko was homozygous for the C1162T mutation (lane 5), and both her mother and father were heterozygous (lanes 6 and 7). A daughter was also heterozygous (lane 8). Another child (data not shown) was also heterozygous. As controls, lane 2 shows common Kell DNA not treated with Tsp45I, lane 3 has untreated Portugal Ko DNA, and lane 4 has common Kell DNA incubated with Tsp45I.

Ko with a Homozygous G2147A (S676N) Mutation—DNA sequence of the Israel KEL gene showed a homozygous G2147A (S676N) mutation in exon 18. To confirm the point mutation determined by DNA sequence, RFLP analysis was performed.

| Ko                 | Nucleotide substitution | Mutation             | Zygosity |
|--------------------|-------------------------|----------------------|----------|
| Michigan Ko        | C502T, exon 4           | R128Stop             | Homozygous |
| North Carolina Ko  | C502T, exon 4           | R128Stop             | Homozygous |
| Yugoslavia Ko      | C366A, exon 4           | C83Stop              | Homozygous |
| Portugal Ko        | C1162T, exon 9          | Q348Stop             | Homozygous |
| Israel Ko          | G2147A, exon 18         | S676N                | Homozygous |
| Reunion Island Ko  | G to A, 5′ intron 3     | Alternative splicing | Homozygous |
| Seattle Ko         | G1208A, exon 10         | S363N                | Heterozygous |
|                    | G to A, 5′ intron 3     | Alternative splicing | Heterozygous |
| New York Ko        | G1208A, exon 10         | S363N                | Heterozygous |
|                    | C694T, exon 6           | R192Stop             | Heterozygous |

Summary of Ko (null) genotypes

**TABLE III**

| Ko                 | Nucleotide substitution | Mutation             | Zygosity |
|--------------------|-------------------------|----------------------|----------|
| Michigan Ko        | C502T, exon 4           | R128Stop             | Homozygous |
| North Carolina Ko  | C502T, exon 4           | R128Stop             | Homozygous |
| Yugoslavia Ko      | C366A, exon 4           | C83Stop              | Homozygous |
| Portugal Ko        | C1162T, exon 9          | Q348Stop             | Homozygous |
| Israel Ko          | G2147A, exon 18         | S676N                | Homozygous |
| Reunion Island Ko  | G to A, 5′ intron 3     | Alternative splicing | Homozygous |
| Seattle Ko         | G1208A, exon 10         | S363N                | Heterozygous |
|                    | G to A, 5′ intron 3     | Alternative splicing | Heterozygous |
| New York Ko        | G1208A, exon 10         | S363N                | Heterozygous |
|                    | C694T, exon 6           | R192Stop             | Heterozygous |

µg/ml cyclohexamide (Sigma) for various periods of time up to 24 h. Immunoprecipitation and endonuclease H treatment of Kell was performed as previously described (36). Briefly, 293T cells were lysed as described above for the isolation of cell surface-exposed protein. A polyclonal antibody to Kell at 1:200 dilution was added and incubated overnight at 4 °C. The immune complex was isolated with protein A-Sepharose, and the proteins were eluted with SDS-loading buffer and separated as described above. The Kell immune complex was treated with endonuclease H (New England BioLabs) as recommended by the manufacturer. The control samples underwent the same procedure except that the enzyme was not added. Protein radioactivity was detected by autoradiography, and the relative amounts were measured by densitometric analysis of the individual bands on the film.

Endothelin-3-converting Enzyme Assay

Red cells were incubated with big endothelin-3 as substrate, and the amount of endothelin-3 produced was measured by enzyme immunoassay as previously described (11).
The G2147A mutation abolishes an AluI site. As expected, a 174-bp PCR-derived sample from wild type DNA yielded two fragments of 137 and 37 bp (Fig. 2, lane 4), while the 174-bp PCR product from the G2147A mutant remained uncut (Fig. 2, lane 5). The 37-bp band (lane 4) is faint and difficult to detect. Lanes 2 and 3 in Fig. 2 are controls showing the PCR products that were not subjected to AluI digestion.

5' Splice Site Mutation in Intron 3—Seven Ko persons from five families living on Reunion Island were studied. DNA sequencing of the 19 exons and the splice site junctions of KEL of one of the subjects was performed, and a homozygous single point mutation (G to A) at the 5'-end of intron 3 was found. This mutation changes the conserved GT sequence to AT, thus disrupting the splice donor site. The same mutation was found in KEL of the other six Ko persons who reside on Reunion Island (data not shown).

Heterozygous Mutations—Two unrelated persons, one from New York and the other from Seattle, were doubly heterozygous for mutations leading to the Ko phenotype. In each case, one of the alleles had a G1208A mutation that substitutes serine at amino acid residue 363 with asparagine. The second Ko allele was different in each case. One Ko (New York) had a C694T mutation in exon 6 resulting in a premature stop codon instead of arginine at amino acid residue 192. The other Ko (Seattle) had G to A mutation in the 5' splice site of intron 3, identical to the Reunion Island mutation, which, as described below, leads to alternative splicing that creates a premature stop codon and exon skipping.

Heterozygosity was first determined by obtaining two peaks at a particular nucleotide position upon direct DNA sequencing of the appropriate PCR products. Both Seattle and New York had G and A present at nt 1208 in exon 7. Seattle also had G and A at a 5' splice site in intron 3, while New York had C and T at nt 694 in exon 6. Confirmation was obtained by subcloning the PCR products and sequencing individual clones. The individual clones contained either the wild type sequence or the mutation.

Alternative Splicing and Exon Skipping—To determine if the G to A mutation at the 5' splice site of exon 3 in KEL of the Seattle Ko causes aberrant splicing, RNA was isolated from the index case and a control subject and reverse transcribed, and the region encompassing nt 54 in exon 1 to nt 823 in exon 7 was amplified by PCR. The expected PCR product of 769 bp was obtained from the wild type cDNA, indicating that both alleles contained the normal sequence (Fig. 3, lane 2). This was confirmed by DNA sequencing. By contrast, two other PCR products of 694 and 638 bp were obtained from Seattle Ko DNA (Fig. 3, lane 3). These 694- and 638-bp products were isolated, subcloned, and sequenced. The 694-bp DNA contained the normal sequence of exon 2 and a partial sequence from exon 3 connected to exon 4. This alternate splicing utilizes a GC sequence in exon 3 as a splice donor site, changes the coding frame, and introduces a premature stop codon in exon 4 (see sequence A in Fig. 4). DNA sequencing of the 683-bp PCR product indicated that exon 3 was skipped, resulting in a frameshift (see sequence B in Fig. 4).

Mutations in Family Members of Seattle Ko—Two sisters, a half-sister, and a daughter of Seattle Ko were genotyped for KEL mutations. Mutations were determined by PCR-RFLP and confirmed by direct DNA sequencing of PCR-amplified products of the appropriate KEL segment. In addition to the proband, one sister was determined serologically to be a Ko and was also a double heterozygote for the G1208A and the 5' intron splice junction mutations. Another sister, the half-sister, and the daughter were all heterozygous for the G to A mutation at the 5' splice junction of intron 3 but were homozygous wild type at nucleotide 1208 (data not shown).

Absence of Kell Protein on Ko Red Cell Membranes—Western immunoblotting, using a polyclonal antibody to Kell protein, was used to analyze red cell membrane proteins. Nearly equal amounts of protein from wild type Kell red cells and from four of the Ko individuals (two with different homozygous premature stop codons and two heterozygotes) were analyzed (Fig. 5).
Fig. 4. Exon skipping and alternative splicing caused by G to A mutation at the 5’ splice site in intron 3. A diagram of KEL exons 2–5 and the intervening introns is shown. The location at the 5’ site in intron 3 at which the G to A mutation in Seattle and Reunion Ko changes the conserved GT sequence to AT is marked. The two types of splice mutants detected (Fig. 3, lane 3, 694- and 638-bp products) are illustrated. Partial sequences that cover the splice sites, obtained from the 769-, 694-, and 638-bp PCR products, are shown. Sequence A is from the 694-bp PCR product (Fig. 3) and shows the utilization of a GC sequence as a splice site in exon 3, changing the reading frame and introducing a stop codon in exon 4. Sequence B is from the 638-bp PCR product (Fig. 3) showing the skipping of exon 3 and the resulting frameshift. Sequence C is from the 769-bp PCR product (Fig. 3, lanes 2 and 3) and shows the normal junction of exons 3 and 4.

Although the blood samples were shipped from different parts of the world and had been stored for various periods of time, there was little evidence of protein degradation as determined by Ponceau S staining (data not shown). Upon separation of the proteins with reduced SDS-PAGE, a polyclonal antibody to Kell protein only detected Kell protein in red cell membranes from wild type Kell (Fig. 5, lanes 4 and 5) and not from any of the Ko samples (Fig. 5, lanes 1, 2, 3, and 6). There was no indication of the presence of truncated Kell proteins in red cells from the Ko persons with premature stop codons, which, if expressed, would be ~14 kDa for Michigan Ko and 38 kDa for Portugal Ko. As a representative of the other Ko red cells studied by Western blots, the Seattle red cell proteins, upon separation by nonreduced SDS-PAGE, lacked or had greatly reduced amounts of the Kell-XK complex, as measured both by antibodies to Kell (Fig. 5, lane 8) and to XK proteins (Fig. 5, lane 10). The faint reaction in the Ko sample at the location of the Kell-XK complex noted with the antibody to XK has not been characterized, but it may be due to a slight cross-reaction of the antibody with another protein or to the presence of XK aggregates. As previously described by others (9), the Ko red cells contained a reduced amount of free XK protein (Fig. 5, lane 10).

Lack of Endothelin-3-converting Enzyme Activity—Red cells from a homozygous Ko person with a premature stop codon (Portugal) and from a heterozygote containing mutations encoding both an amino acid substitution and a splice-junction defect (Seattle) were analyzed for endothelin-3-converting enzyme activity. As previously shown for another Ko individual (11), and in contrast to red cells of normal Kell phenotype, the Ko red cells lacked endothelin-3-converting enzyme activity (data not shown).

The S363N and S676N Mutations Inhibit Protein Trafficking to the Cell Surface—Red cells of Ko persons with serine to asparagine substitutions at exons 10 (S363N) and exon 18 (S676N) do not contain Kell protein. To determine the mechanism by which these amino acid substitutions prevent expression of the mutant Kell proteins on the cell surface, expression vectors were constructed containing these mutations, and their expression by 293T cells was compared with that of wild type Kell. As a further control, a cDNA was prepared that encoded asparagine instead of serine at amino acid position 650, a reaction in the Ko sample at the location of the Kell-XK complex are marked.

For the S676N mutant were detected on the cell surface (Fig. 6B, lanes 2 and 3). The Kell proteins with S363N or S676N mutations were not detected on the cell surface (Fig. 6B, lanes 4 and 5).
domain present in Kell, a type II membrane glycoprotein (41, 42). With these mutations, a truncated protein may be expressed and initially inserted into the ER membrane, but in no case was there any evidence of the presence of a truncated protein on the red cells of the Ko individuals, indicating that the truncated proteins are probably degraded intracellularly and are not transported to the cell surface membrane. Interestingly, a homozygous C502T mutation encoding an R128Stop codon was found in two unrelated persons, both of whom shared a KEL6 genotype. The KEL6 antigen has an approximate incidence of 20% in persons of African heritage and has a negligible incidence in persons of European heritage (43), and both of these Ko individuals were African-Americans. While coincidence cannot be ignored, this raises the possibility that the C502T mutation may be more common among KEL6 individuals than those with the KEL6 phenotype. The C502T mutation would remain undetected if it exists in a heterozygous condition together with a wild type allele, since the majority of Ko persons are detected because they have produced a Kell-related antibody, usually in response to pregnancy or blood transfusion.

The second type of point mutation that leads to the Ko phenotype is disruption of the conserved GT sequence at the 5' splice junction in intron 3. A homozygous G to A mutation was found in four different families, all residing on Reunion Island in the Indian Ocean, where intermarriage has been frequent in the past. This mutation was also present, in a heterozygous state, in several members of a family from the Pacific Northwest in the United States. A point mutation at the same splice site in intron 3, but G to C instead of G to A, was reported for a Ko person from Taiwan (31). Both of these mutations cause skipping of exon 3, which contains the trans-membrane domain, and introduction of a downstream premature stop codon. We have also shown, by reverse transcriptase-PCR analysis, that additional alternative splicing occurs in which a GC sequence in exon 3 is used as a splice donor site, changing the coding frame and introducing a premature stop codon in exon 4 (see Fig. 4). Although unusual, several GC splice site variants have been described (44).

Point mutations leading to amino acid substitutions are common in the Kell blood group system and are the cause of the different Kell red cell phenotypes (3). In most cases, the amino acid substitutions do not affect the number of copies of Kell protein on the red cell membrane but merely present different cell surface epitopes. There is, however, an example, KEL3, in which an R281W substitution leads to a reduced amount of Kell protein on the red cell membrane (45). We now show that S363N and S676N mutations have a more severe effect and lead to a condition in which the levels of Kell protein on the cell surface membrane are not detectable by serologic procedures or lead to a condition together with a wild type allele, since the majority of Ko persons are detected because they have produced a Kell-related antibody, usually in response to pregnancy or blood transfusion.

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with asparagine in Kell increases the homology with the other family members but, parenthetically, inhibits trafficking to the plasma membrane.

Although the Ko phenotype is rare, the fact that two unrelated Ko persons were heterozygotes suggests that the individual mutations may be more frequent than previously thought. Both Seattle Ko and New York Ko have identical G1208A (S363N) mutations in one allele. They differ in the second mutation; New York has a unique C694T (R192Stop) mutation in exon 6 in the other allele, while Seattle shares the same 5′ splice junction mutation in intron 3 as that found in the Ko persons from Reunion Island. As mentioned earlier, the presence of any one of these mutations in a heterozygous state together with a wild type allele will probably remain undetected.

Kell is a member of the neprilysin family of zinc endopeptidases, whose principal function is the activation of bioactive peptides by proteolytic cleavage of larger inactive polypeptides (12). While this family of enzymes has distinct substrate specificity, there is overlap in function, especially between Kell and ECE-1 and ECE-2. Big endothelin-3 is the preferred substrate for Kell, and it is nearly 100 times more effective as a substrate than big endothelin-1 and big endothelin-2. However, Kell does activate endothelin-1 and endothelin-2. Conversely, ECE-1 and ECE-2 prefer big endothelin-1 as substrate but can also cleave big endothelin-2 and -3. The M13 family has strong amino acid

**FIG. 7.** S363N Kell mutant is degraded more rapidly than wild type Kell. 293T cells expressing wild type Kell and the S363N mutant were pulse-labeled with L-[35S]methionine for 20 min and chase-incubated for various periods of time up to 24 h. Degradation was measured by determining the percentage of radioactive Kell protein remaining at the chase periods. The data are from two separate experiments.

**FIG. 8.** Endonuclease H treatment of wild type and S363N Kell proteins. 293T cells expressing wild type Kell and the S363N mutant were pulse-labeled with L-[35S]methionine for 20 min and chase-incubated for 3.5 and 5.5 h. Kell protein was isolated at the end of the pulse and chase periods, treated with endonuclease H, separated by SDS-PAGE, and detected by autoradiography. Lanes 1–4 contain proteins isolated at the end of the pulse period. Lanes 5–8, proteins isolated after 3.5 h of the chase period; lanes 9–12, proteins isolated after 5.5 h of the chase period. Wild type (wt) Kell are shown in lanes 1, 2, 5, 6, 9, and 10, and the S363N mutant is shown in lanes 3, 4, 7, 8, 11, and 12. Isolated proteins were either treated (+) or not treated (−) with endonuclease H (Endo H).

**FIG. 9.** Clustal alignment at exon 10 of Kell and other members of the neprilysin family of zinc endopeptidases. A ClustalV alignment analysis of Kell, ECE-1, neutral endopeptidase (NEP), and the product of the PEX gene is shown. The location of the S363N mutation, present in New York and Seattle Ko, is noted with a circle on the affected serine residue. Parenthetically, a S363N substitution enhances the homology at this location.
sequence homology at the C-terminal domain that coincides approximately with residues 550–732 of Kell (1, 2). It is within this region that the enzyme active sites reside. In all of the Ko persons studied who had premature stop codons, the stop signal preceded the conserved region, thus assuring that any truncated protein expressed will lack enzyme activity. Since there were no obvious common clinical conditions noted in these Ko persons, our results suggest that a lack of Kell enzyme activity does not result in a recognizable illness. Compensatory mechanisms may be activated in the Ko phenotype, probably involving the overlapping functions of the nephrilysin family of enzymes.

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