Association of XK and Kell Blood Group Proteins*

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A disulfide bond links Kell and XK red cell membrane proteins. Kell, a type II membrane glycoprotein, carries over 20 blood group antigens, and XK, which spans the membrane 10 times, is lacking in rare individuals with the McLeod syndrome. Kell is classified in the neprilysin family of zinc endopeptidases, and XK has structural features that suggest it is a transport protein. Kell has 15 extracellular cysteines, and XK has one in its fifth extracellular loop. Five of the extracellular cysteine residues in Kell are not conserved in the other members of the neprilysin family, and based on the hypothesis that one of the nonconserved cysteines is linked to XK, cysteines 72 and 319 were mutated to serine. The single extracellular cysteine 347 of XK was also mutated. Co-expression of combinations of wild-type and mutant proteins in transfected COS-1 cells showed that Kell C72S did not form a Kell-XK complex with wild-type XK, while wild-type Kell and Kell C319S did. XK C347S was also unable to form a complex with wild-type Kell, indicating that Kell cysteine 72 is linked to XK cysteine 347. Kell C72S was transported to the cell surface, indicating that linkage to XK is not required. In addition, chemical cross-linking of red cell membranes with dithiobispropionimidate indicated that glyceraldehyde-3-phosphate dehydrogenase is a near neighbor of Kell.

The association of Kell and XK proteins on red cell membranes was predicted from early serological studies on two rare Kell-related phenotypes that noticed a relationship between antigens residing on the Kell blood group protein and Kx antigen, which is carried on a separate XK protein. In the McLeod phenotype red cell, there is an absence of Kx, an otherwise ubiquitous red cell antigen, and this is accompanied with a depression of all Kell antigens. On the other hand, in the Ko (null) phenotype, in which there is no detectable Kell surface antigen, there is an enhanced level of Kx antigens (1, 2). Also, treatment of normal red cells with reducing reagents, which inactivate the Kell antigens, showed a marked increase in Kx activity, indicating a sulfhydryl involvement in the presentation of Kell and Kx antigens on the red cells (3). Biochemical studies in which Kell protein was isolated from red cells in nonreduced conditions, showed that Kell protein was associated with itself as an oligomer and was also complexed with other red cell membrane proteins (4). Later, immunoprecipitation of Kell protein in nonreduced conditions co-isolated XK, demonstrating that the two proteins exist as a disulfide-bonded complex on the red cell membrane (5). Further studies on the Kell-XK complex showed its absence in McLeod red cells and also demonstrated that although Ko (null) red cells have high Kx activity they contain less XK protein, indicating that lack of Kell protein may expose more Kx antigens on the cell surface (6).

The McLeod phenotype has an X-linked mode of inheritance, and since some McLeod patients also have chronic granulomatous disease and/or Duchenne muscular dystrophy, XK was located close to those genes at the Xp21 region of the X chromosome (7). The red cell membrane protein (XK) that carries Kx antigen migrates on SDS-PAGE as a 37-kDa polypeptide (8). Positional cloning isolated the XK gene and predicted that it encodes a 444-amino acid protein that spans the red cell membrane 10 times and has structural characteristics of a membrane transporter (9). Since XK protein may only have one extracellular cysteine residue in the fifth extracellular loop, it was predicted that this residue may be involved in disulfide linkage with Kell protein, which has 15 extracellular cysteine residues and one cysteine in the transmembrane domain (5, 10).

McLeod red cells, which lack XK protein, have abnormal cell shape with a high proportion of acanthocytes. Another striking aspect of the McLeod phenotype is its association with a late onset form of muscular dystrophy, although high serum levels of the muscle enzyme, creatine phosphokinase are noted at earlier ages (11). Some McLeod patients also develop neurological symptoms (1, 2, 12). In accordance with the clinical studies, XK transcripts are present not only in erythroid tissues but also in skeletal muscle, brain, heart, and pancreas (9). This is in contrast to KEL, which appears to be mostly restricted to erythroid tissues (13). Molecular cloning of KEL (14) showed that the Kell protein is a 731-amino acid type II membrane glycoprotein with close structural and sequence similarities to a subfamily of zinc endopeptidases that includes neutral endopeptidase 24.11, endothelin-converting enzyme, and the product of the PEX gene (10, 15–19).

In this study, the association of Kell and XK on red cell membranes is explored with emphasis on the topology of XK, on the near neighbor relationship of Kell to other red cell membrane proteins, and on the cysteine residues that link Kell and XK proteins.

MATERIALS AND METHODS
Antibodies
A 42-amino acid synthetic peptide, corresponding to the second extracellular loop domain of XK, was prepared by Synpep (Deblin, CA). A 30-mer peptide derived from an intracellular domain of Kell (amino acids 2–31) was synthesized by the Microchemistry Laboratory of the New York Blood Center. Polyclonal antibodies were raised in rabbits and were affinity-purified. The antibody to the XK peptide reacted with several red cell membrane proteins on Western immunoblots, but it isolated XK from a nonionic detergent extract from normal, but not from McLeod, red cells. The antibody to the Kell peptide was specific.

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both by Western immunoblotting and the ability to isolate Kell from a red cell detergent extract. Polyclonal antibody to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH),\(^1\) purchased from Sigma, was raised in mice. A mouse monoclonal antibody to a Kell surface antigen (KEL14) was a gift from Dr. Pablo Rubinstein of the New York Blood Center.

**Immunoprecipitation of Kell-XK Complex**

**Red Cells—Surface-exposed Kell** was isolated from red cells using a monoclonal antibody to KEL14. Red cells were coated overnight with 5 volumes of 300 µg/ml anti-KEL14 at 4 °C. The cells were washed with phosphate-buffered saline, and membrane “ghosts” were prepared as described previously (4). The membrane were solubilized with 1% Triton X-100, 0.5% sodium deoxycholate in phosphate-buffered saline containing protease inhibitors, and the solution was cleared by centrifugation at 27,000 rpm for 20 min in a Beckman 50Ti rotor. The following protease inhibitors were used: 0.1 mM 1-1-tosylamido-2-phenyl ethyl chloromethyl ketone, 0.1 mM phenylmethylsulfonyl fluoride, and 10 units/ml aprotinin (Sigma). The Kell-antibody complex was isolated with protein G coupled to Sepharose (Fierce) as described previously (4, 20).

XK and XK-Kell complexes were also isolated from detergent-soluble extracts of red cell membranes using rabbit antibody to a peptide representing the second loop of XK. Membrane ghosts were prepared and solubilized as described above and treated overnight at 4 °C with 3.5 µg/ml affinity-purified antibody. The immune complex was isolated with protein A-Sepharose.\(^2\)

**Transfected COS Cells—**Confluent, transiently transfected, COS cells were incubated with L-[\(^35\)S]methionine (NEN Life Science Products; specific activity, 1110 Ci/mmol) in 100-mm Petri dishes. After removal of the incubation medium, the cells were lysed with 1 ml of 0.5% sodium deoxycholate, 1% n-dodecyl-\(\beta\)-maltoside (Sigma), and a mixture of protease inhibitors described above. The extract was centrifuged in a microcentrifuge at 12,000 rpm for 10 min at 4 °C. Antibody to the second loop of XK (3.5 µg/ml) or to the COOH-terminal peptide of Kell (3.5 µg/ml) was added and incubated overnight at 4 °C. The immune complex was isolated with protein A-Sepharose.

To isolate surface-exposed Kell and its complexes from transfected COS cells, the cells were labeled overnight with [\(^35\)S]methionine, and the cells, attached to the bottom of the 100-mm Petri dishes, were washed with phosphate-buffered saline and then treated for 30 min over ice with 3 ml of 300 µg/ml mouse monoclonal antibody to KEL14. The cells were again washed with phosphate-buffered saline and lysed with detergent as described above. The immune complexes present in the detergent-soluble extract were isolated with protein G-Sepharose.

**Separation of Kell-XK Complexes by SDS-Polyacrylamide Gel Electrophoresis**

The immune complexes on protein A or protein G-Sepharose were eluted with SDS-loading buffer (0.25 M Tris-HCl, pH 6.8, 1% SDS, 5% glycerol or 4 M urea with or without 10 mM dithiothreitol or 300 mM 2-mercaptoethanol, 0.005% bromphenol blue) and separated by SDS-PAGE using 9% polyacrylamide gels.

**Chemical Cross-linking of Kell to Neighboring Proteins**

Red cells were coated with monoclonal antibody to KEL14, washed with normal saline, and lysed with 5 mM phosphate buffer, pH 7.4, and membrane “ghosts” were prepared as described previously (4). The washed membranes, suspended in 5 volumes of 25 mM sodium phosphate, pH 8, containing 1 mM MgCl\(_2\), were cross-linked at room temperature for 2 h with a final concentration of 4 µg/ml freshly prepared 3,3′-dithiobispropionimidate (DTBP) purchased from Pierce. The membrane complexes were solubilized with 0.5% sodium deoxycholate and 1% Triton X-100 and the proteins, cross-linked to the Kell antibody complex, were isolated with protein G-Sepharose. Since DTBP is a thiol-cleavable cross-linking reagent, the isolated complexes were disassembled with DTT, separated on SDS-PAGE, detected by silver staining, or analyzed by Western immunoblotting using polyclonal antibody to GAPDH or to Kell protein.

1. The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide electrophoresis; PCR, polymerase chain reaction; COS-1, monkey kidney fibroblast cells; CMV, cyto megalovirus; HPLC, high pressure liquid chromatography; kb, kilobase pair; bp, base pair.

**Amino Acid Sequencing**

Proteins, separated by SDS-PAGE, were transferred to ProBlot\(^\text{TM}\) polyvinylidene difluoride membranes (purchased from Applied Biosystems, Foster City, CA) and digested with trypsin (21). The released peptides were separated by HPLC, and well separated peptides, were sequenced using an automated Applied Biosystems, model 477A, amino acid sequencer.

**Site-directed Mutations and Construction of Expression Vectors**

All site-directed mutations were carried out by PCR, changing a single base in the cysteine codon of one of the primer sets to encode serine. The primers containing the mutation site included a restriction enzyme site that, used together with another nearby unique restriction enzyme site in the cDNAs of Kell or XK, were used to replace the respective region of the wild-type DNA. The wild-type cDNAs were placed in the expression vector pBAM (Invitrogen Inc.) and were linearized before being used as template DNA in the PCR procedure. When there was more than a single restriction enzyme site in the construct, limited digestions were performed.

Positive clones were selected with the appropriate restriction enzyme or by sequencing if it was not possible to select with restriction enzymes. The mutated regions in the plasmids were confirmed by sequencing.

**Construction of Kell and XK Expression Vectors**

An expression vector, pRC/CMV, containing XK and Kell cDNAs, either wild-type or mutant, in tandem, each with its own CMV promoter and polyadenylation signals, was constructed as summarized in Fig. 1. Kell cDNA was modified to contain a Kozak sequence (22) before the ATG initiation codon as follows. A Kozak sequence (underlined) was placed at the 5′-end of a sense PCR primer, and an antisense primer was modified to contain a BamHI site (also underlined). These primers were used to amplify full-length Kell cDNA.

**Polymerase Chain Reaction (PCR)**

The following denaturation, annealing, and polymerization steps were performed in an automated thermocycler (Minicycler; MJ Research Inc, Watertown, MA). The initial cycle was 94 °C for 3 min, 60 °C for 1 min, and 72 °C for 30 s; in cycles 2 through 30, the conditions were 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. In the last cycle, the polymerization step at 72 °C was extended to 7 min to complete copies.

**Kell C72S**

The PCR procedure described earlier was used to create a G353C mutation in Kell cDNA that encodes serine instead of cysteine at Kell amino acid 72. The PCR product was inserted into wild-type Kell cDNA that had been modified to contain a Kozak sequence (GGGCGCAC) before the ATG initiation codon, as described above, and had been placed in pRC/CMV vector using HindIII and XhoI cloning sites. Wild-type Kell in pRC/CMV was amplified using the following primers to yield a 512-bp product.

**Sense primer:** 5′-GGGGTGGCATCTTTGGGATCCAAGTTACC-3′

**Anti-sense primer:** 5′-GGGGTGGCATTCTTTGGGATCCAAAGTACC-3′

The 2.2-kb PCR product was placed in pBC SK(+) vector (Stratagene, La Jolla, CA) using EcoRV and BamHI cloning sites. A 650-bp fragment was released by cutting with HindIII and PpuMI (at nucleotide 753 of Kell cDNA) and ligated to a 7-kb Kell cDNA in pRC/CMV expression vector that had been cut with HindIII and PpuMI.

**BstXI**

The PCR product described earlier was used to create a G353C mutation in Kell cDNA that encodes serine instead of cysteine at Kell amino acid 72. The PCR product was inserted into wild-type Kell cDNA that had been modified to contain a Kozak sequence (GGGCGCAC) before the ATG initiation codon, as described above, and had been placed in pRC/CMV vector using HindIII and XhoI cloning sites. Wild-type Kell in pRC/CMV was amplified using the following primers to yield a 512-bp product.

**Sense primer:** 5′-GGGGTGGCATCTTTGGGATCCAAGTTACC-3′

**Anti-sense primer:** 5′-GGGGTGGCATTCTTTGGGATCCAAAGTACC-3′

The sense primer contained a G to C mutation that is conveniently cut with BstXI at the mutation site (underlined). The PCR product also contains a PpuMI site that is unique in Kell cDNA. Kell cDNA in pRC/CMV, however, has two BstXI sites (nucleotides 165 and 327). First, pRC/CMV Kell was cut with PpuMI followed by limited digestion with BstXI. A 7.5-kb band that was produced was isolated by 0.5% low melting agarose gel electrophoresis. The 512-bp PCR product was also cut with BstXI and PpuMI, and the isolated 427-bp product was ligated to the 7.5-kb Kell cDNA fragment. The ligation products were transformed into Top10F\(^\text{TM}\) Escherichia coli competent cells (Invitrogen). Miniplasmid preparations were performed using RPMII Miniplasmid preparation kit (BIO 101, Inc., Vista, CA). Positive clones were selected,
and plasmid DNA was obtained by the polyethylene glycol procedure.

**Kell C319S**

A similar procedure was followed to create the Kell C319S mutant. The following primers were used to create a 224-bp PCR product containing the G1076C mutation.

**Sense primer:** 5'-GCCCGCCGGATGCTGGAT-3'  
Anti-sense primer: 5'-GGCTTTCTGCGTGCCTCCTGGAATTGAC-3'

An AhdI site in the sense primers is underlined, as is the G to C mutation. Template pRC/CMV-Kell was used, and the PCR product was cut with AhdI and BstEII and purified in 1% low melting agarose electrophoresis. Kell in pRC/CMV was cut with BstEII, followed by limited digestion with AhdI. A 7.6-kb fragment was isolated and ligated with the 176-bp PCR product.

**RESULTS**

*Anti-body to XK Co-isolates Kell Protein from Normal but Not from McLeod Red Cells—Previous studies showed that Kell and XK are surface-exposed on red cells, and both can be labeled by radioactive iodine (4, 8). Normal and McLeod red cells were surface-labeled with 125I by the lactoperoxidase-catalyzed method (4, 23) and lysed, and the washed membranes were solubilized with detergents. Rabbit antibody to a synthetic peptide, which represents the predicted second extracellular loop of XK protein, was used to immunoprecipitate radioactive XK and any other associated surface-labeled proteins. The isolated proteins were separated by SDS-PAGE in reduced conditions.

The patterns of total surface-labeled proteins in normal and McLeod red cells were similar. The majority of radioactivity corresponded with the predominant surface-exposed proteins, band 3 and the glycophorins (Fig. 2, lanes 1 and 3). Antibody to the second loop of XK isolated radioactive proteins from normal red cells (Fig. 2, lane 4) but not from McLeod red cells that lack this protein (Fig. 2, lane 2). From normal red cells, radioactive proteins were noted at the top of the SDS-PAGE gel and at two other locations that correspond to proteins of approximately 93 and 40 kDa. The electrophoretic migration of the 93- and 40-kDa proteins correspond to the expected sizes of Kell and XK proteins.

Failure to isolate the 40-kDa protein from extracts of McLeod red cells and its presence in normal cells indicated that the 40-kDa protein is surface-exposed XK protein. Western immunoblot using a rabbit antibody specific for Kell protein, reacted with the 93-kDa protein (data not shown). The 93-kDa protein was transferred from the SDS-polyacrylamide gels to polyvinylidene difluoride membranes and treated with trypsin, the peptides were separated by HPLC, and a well separated peptide was sequenced. The sequence VSPWDYNAYYSVSD corresponds with amino acid residues 535-552 of Kell protein (14).

**GAPDH Is a Near Neighbor of Kell Protein on the Red Cell Membrane—To determine if other proteins are associated with or are near neighbors of Kell, red cells were treated with a mouse monoclonal antibody to KEL14, which specifically reacts

**Transient Transfection of COS Cells**

About 2 x 10⁷ cells were released from Petri plates with trypsin-EDTA, washed twice with phosphate-buffered saline, and suspended for 15 min with 10 μg of the circular plasmid DNA prior to electroporation. Electroporation was performed in 1 ml of 1x electroploration media (Life Technologies, Inc.) to which an electrical pulse (250 V, 330 microfarads) was applied in a Life Technologies cell porator. The cells were allowed to recover for 10 min in ice and were then seeded at 5 x 10⁶ cells on 100-mm Petri dishes with 12 ml of growth medium (RPMI 1640 supplemented with 10% fetal bovine serum) and grown to confluency in 2 days.

**Metabolic Labeling of COS Cells with L-[35S]Methionine**

Two days after transfection, confluent COS cells (100-mm plates) were washed with phosphate-buffered saline and incubated at 37°C for 30 min or overnight in 1 ml of L-methionine-free medium with 0.5 mCi of L-[35S]methionine. The cells were then washed twice with phosphate-buffered saline and "chase" incubated with normal growth media for 5 h.
mutated proteins were co-expressed in COS cells, and their residues in XK and two different cysteines in Kell were mutated. The wild-type or the residue does not affect Kell-XK formation and that Kell Cys72 specifically links Kell to XK. Co-isolation of Kell protein with antibody to XK. Normal (common phenotype) and McLeod red cells were surface-labeled with 125I, and proteins were isolated from a detergent extract by immunoprecipitation with an antibody to XK. Radioactive proteins were separated by reduced SDS-PAGE and detected by autoradiography. Lanes 1 and 3 contain total membrane proteins. The locations of band 3 and glycophorin A (GPA) are marked. Lane 1, normal red cells; lane 3, McLeod red cells. Lanes 2 and 4 have proteins isolated by immunoprecipitation. Lane 2, proteins from McLeod red cells; lane 4, proteins from normal red cells. The locations of Kell and XK are marked.

The silver-stained protein patterns are shown in Fig. 3. In dithioisopropionimidate-cross-linked membranes (Fig. 3, lane 1), but not in untreated membranes (Fig. 3, lane 2), a 36-kDa protein co-precipitated with Kell protein. In these experiments, very little XK was detected, and the predominant silver-stained proteins were Kell and the heavy and light chains of IgG. The bands of approximately 30 and 24 kDa, that appear to be more prominent in the cross-linked sample in this experiment, were variable and were not characterized. The 36-kDa protein was unique in the cross-linked samples and was identified as GAPDH by amino acid sequencing of two peptides isolated from a trypsin digest. Two peptides, separated by HPLC, had the amino acid sequences LVSWYDNEFGYSNR and GALQXIFT.

These two sequences correspond to amino acid residues 19–32 and 200–214 of human GAPDH. The 36-kDa protein also reacted on Western immunoblotting with a rabbit antibody to human GAPDH (data not shown).

Identification of Cysteine Residues Involved in Kell-XK Disulfide Linkage—Hydropathy plots predict that XK has a single extracellular cysteine residue in its small, 11-amino acid, fifth loop (9) and that Kell has 15 extracellular cysteine residues (14). Any one of the 15 extracellular cysteine residues of Kell could form a disulfide bond with the single extracellular cysteine of XK. To determine which of the cysteine residues are involved in disulfide linkage, the single cysteine extracellular residue in XK and two different cysteines in Kell were mutated to serine by site-directed mutagenesis. The wild-type or the mutated proteins were co-expressed in COS cells, and their ability to form Kell-XK disulfide-bonded complexes was determined.

Kell is a member of a subfamily of membrane zinc metalloendopeptidases, which includes neutral endopeptidase 24.11, endothelin-converting enzyme, and the product of the PEX gene (14–19, 24). Members of this subfamily of zinc endopeptidases conserve 10 cysteine residues in their extracellular domains. We hypothesized that one of the five nonconserved cysteine residues of Kell is likely to be involved in linkage to XK. A likely candidate was Kell cysteine 72, since it is close to the membrane-spanning domain, as is the single cysteine of XK. As a control, we also mutated cysteine 319 of Kell to serine, since this cysteine residue is the next nonconserved cysteine from the transmembrane domain.

COS cells were transiently transfected with an expression vector containing Kell and XK cDNAs in tandem and the recombinant Kell-XK complex isolated with an antibody to the second loop of XK. On analysis, by SDS-PAGE under reduced conditions, both Kell (93 kDa) and XK (40 kDa) were detected when wild-type XK and Kell proteins were co-expressed (Fig. 4A, lane 1). Kell was not co-isolated with XK, indicating a lack of Kell-XK formation, when the Kell mutant (C72S) was co-expressed with wild-type XK (Fig. 4A, lane 2) or when the XK mutant (C347S) was co-expressed with wild-type Kell (Fig. 4A, lane 4). However, co-expression of XK with another Kell mutant (C319S) did co-isolate Kell protein (Fig. 4A, lane 3), indicating that mutation of another nonconserved Kell cysteine residue does not affect Kell-XK formation and that Kell Cys72 specifically links Kell to XK.

Analysis on nonreduced SDS-PAGE confirmed that a 134-kDa Kell-XK complex was formed when wild-type XK and Kell (Fig. 4B, lane 1) and when wild-type XK and Kell mutant (C319S) (Fig. 4B, lane 3) were co-expressed. However no 134-kDa complex occurred when XK and Kell mutant (C72S) (Fig. 4B, lane 2) or mutant XK (C347S) and wild-type Kell (Fig. 4B, lane 4) were co-expressed. Taken together, these results indicate that Kell Cys72 is linked to XK Cys347.
Although COS cells assemble recombinant Kell and XK, not all of the expressed proteins were disulfide-linked. Free XK was detected with antibody to XK, in conditions that isolated Kell-XK complex (Fig. 4B, lanes 1 and 3), and when antibody to Kell was employed, free Kell was detected, in addition to the 134-kDa complex (Fig. 4C, lane 1).

Both Kell-XK Complex and Free Kell Are on the Cell Surface of Transfected COS Cells—To establish if Kell-XK formation is required for cell surface expression and also to determine the fate of mutant Kell (C72S), COS cells, co-expressing wild-type XK and Kell or wild-type XK and mutant Kell (C72S), were incubated overnight with L-[35S]methionine and treated with an antibody to Kell (anti-KEL14) that recognizes Kell surface antigens. The cells were washed and lysed with detergents, and the radioactive proteins associated with anti-KEL14 were analyzed by nonreduced SDS-PAGE and autoradiography. When wild-type XK and Kell proteins were co-expressed, both the 134-kDa XK-Kell complex and free Kell (93 kDa) were present on the cell surface (Fig. 5, lane 2). Upon co-expression of wild-type XK and mutant Kell (C72S), the 134-kDa complex was not detected on the cell surface, but mutant Kell (C72S) was present (Fig. 5, lane 1). This demonstrates that wild type Kell and XK occur as a complex on the cell surface of transfected COS cells but that Kell does not have to be complexed with XK to be transported to the cell surface.

DISCUSSION

The Kell blood group glycoprotein is classified as a member of the neprilysin family (M13) of zinc metallopeptidases (24). Proteins in the M13 family are type II membrane glycoproteins and currently consist of neutral endopeptidase 24.11, Kell, endothelin-converting enzyme, and the product of the PEX gene. This topic was recently reviewed (15). The physiological substrates of neutral endopeptidase 24.11 and endothelin-converting enzyme are known, but those of Kell and PEX have not yet been identified. Neutral endopeptidase 24.11 is widely distributed and has broad specificity, processing a large number of peptide hormones (15, 25). By contrast, endothelin-converting enzyme is more specific and activates a 38- or 39-amino acid “big” endothelin, by cleavage of a Trp21–Val22 bond, to an active 21-amino acid endothelin peptide (15–17). Endothelin is a potent vasoconstrictor that plays an essential role in maintaining vascular tone. Endothelin-converting enzyme is a dimer consisting of two disulfide-linked 130-kDa subunits. Site-directed mutagenesis and expression in COS cells suggests that the endothelin-converting enzyme subunits are linked by Cys412, a residue not conserved in neutral endopeptidase 24.11, Kell, or PEX (26). In this study, we demonstrate that Kell is covalently linked through a nonconserved Cys72 to Cys347 of XK protein (see Fig. 6). By contrast, neutral endopeptidase 24.11 is not covalently linked with itself or with another protein. Therefore, Kell differs from the other members of the M13 family in that it is linked to another membrane protein. XK, to which Kell is linked, is an integral membrane protein, which probably spans the membrane 10 times and has both its NH2 terminus and COOH terminus within the cell. The function of XK is not known, although it has structural features that suggest a transport function.

Thus, although the functions of both Kell and XK are un-
lack XK (1, 2). In this study, we also show that a specific Kell antigen, KEL14, is expressed on the surface of transfected COS cells, in the absence of XK, demonstrating that Kell protein can be transported to the cell surface without being linked to XK. In addition, since Kell protein, by itself, expresses KEL14, a conformational epitope, these data suggest that Kell protein assumes normal folding in the absence of XK. Since mutant Kell (C72S) was transported to the cell surface of COS cells in the absence of XK and retained its ability to be recognized by a specific monoclonal antibody to the KEL14 conformational epitope, these results further indicate that mutation of Kell (C72S) does not markedly change the tertiary structure of the protein.

Our results are in agreement with previous studies (5, 6) that show that Kell and XK are disulfide-linked in the native state on human red cells. There is no evidence, however, that Kell and XK are part of a larger membrane complex, since antibodies to Kell or XK did not isolate other proteins and since chemical cross-linking studies, using a reagent that detects near neighbors with free amino groups, only co-isolated a cytoplasmic protein, GADPH. Although the cross-linking studies may not be quantitative, it appears that not all of the Kell proteins on red cells are cross-linked to GADPH. GADPH is known to be linked to other red cell membrane proteins, notably band 3, the anion transporter (28).

The hydrophathy plot of XK predicts five extracellular loops and predicts that Cys347 resides on the small fifth extracellular loop (9). Our results demonstrate that Kell cysteine 72, which is known to be in the extracellular domain of Kell protein (27), is linked to XK Cys347. This indicates that the disulfide linkage of Kell to XK lies close to the extracellular membrane surface and supports the predicted topology of XK.

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