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Intracellular assembly of Kell and XK blood group proteins

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Abstract

Kell, a 93 kDa type II membrane glycoprotein, and XK, a 444 amino acid multi-pass membrane protein, are blood group proteins that exist as a disulfide-bonded complex on human red cells. The mechanism of Kell/XK assembly was studied in transfected COS cells co-expressing Kell and XK proteins. Time course studies combined with endonuclease-H treatment and cell fractionation showed that Kell and XK are assembled in the endoplasmic reticulum. At later times the Kell component of the complex was not cleaved by endonuclease-H, indicating N-linked oligosaccharide processing and transport of the complex to a Golgi and/or a post-Golgi cell fraction. Surface-labeling of transfected COS cells, expressing both Kell and XK, demonstrated that the Kell/XK complex travels to the plasma membrane. XK expressed in the absence of Kell was also transported to the cell surface indicating that linkage of Kell and XK is not obligatory for cell surface expression.

Keywords: Kell; XK; Blood group; Red cell; Protein assembly

1. Introduction

Kell is a 93 kDa type II membrane glycoprotein [1] and XK a 444 amino acid integral membrane protein that is predicted to span the membrane 10 times [2]. Both Kell and XK proteins display blood group antigens and on human red cell membranes Kell and XK are linked by a disulfide bond [3,4]. Kell is highly polymorphic, and over 20 different antigens are recognized as part of the Kell blood group system. Some of the Kell antigens are strong immunogens and the Kell blood group system is important in transfusion medicine since Kell antibodies can elicit severe reactions if incompatible blood is transfused [5,6]. Kell antibodies can also suppress erythropoiesis and cause anemic disease in newborn infants [7–9]. The different Kell antigens are due to point mutations in *KEL* leading to single amino acid changes in the extracellular domain of the glycoprotein [10–15]. XK on the other hand, carries a single blood group antigen, termed Kx, which is present in all red cells except in the rare McLeod phenotype. McLeod red cells have aberrant shape, appearing as acanthocytes, and together with lack of XK there is a marked diminution of Kell protein and all Kell antigens are markedly weakened. Lack of XK is associated with disease and most McLeods exhibit a late onset form of muscular dystrophy, cardiomyopathy, and nerve degeneration [5,6,16,17].

Expression of wild-type and mutant recombinant

Abbreviations: NEP, neutral endopeptidase; ECE, endothelin converting enzyme; ER, endoplasmic reticulum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethyl sulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone

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Kell and XK proteins has indicated that Kell Cys^{72}, which is close to the membrane spanning domain, is linked to XK Cys^{347} located in a small 'loop' which contributes the fifth extracellular XK domain [18]. Although Kell and XK exist on red cell membranes as a disulfide-bonded complex, association of the two proteins is not needed for presentation of Kell blood group antigens. Expression of Kell protein in a variety of transfected cells indicates that Kell can be transported to the plasma membrane without co-expression of XK and the recombinant protein expresses Kell surface antigens [15,19]. Likewise, Kx antigen is present in Ko (null) red cell membranes which have little, or no, Kell protein [5,6]. It is not known, however, whether the association of Kell and XK is needed for red cell functions, since the complete physiological roles of the two proteins remain to be determined. Kell protein has close structural and sequence homology to the M13 or neprilysin sub-family of zinc endopeptidases [15] which include neutral endopeptidase 24.1 (NEP), endothelin converting enzyme (ECE) and the product of the *PEX* gene [20–24]. The members of the M13 family of membrane zinc endopeptidases are involved in the activation and inactivation of biologically active peptide hormones. Recently we determined that the extracellular domain of Kell protein, like ECE-1, activates the endothelins, but that unlike ECE-1 Kell preferentially activates endothelin-3 rather than endothelin-1 [25]. Although absence of XK is associated with red cell acanthocytosis and with muscle and nerve disorders and its primary structure has features that suggest that it may be a membrane transporter [2], its function remains to be determined.

To study the mechanism of Kell/XK assembly we have, using co-expression of Kell and XK in COS cells, determined the biosynthesis, site of assembly and intracellular transport of Kell and XK proteins.

2. Materials and methods

2.1. Antibodies

A 42-mer synthetic peptide, corresponding to the predicted second extracellular loop of XK was prepared by Synpep, Deblin, CA, USA and a 30-mer peptide representing the N-terminal domain of Kell protein (amino acids 2 to 31) was synthesized by the Microchemistry Laboratory of the New York Blood Center. Polyclonal antibodies were produced in rabbits by injection of the synthetic peptides mixed with Freunds adjuvant and the antisera were affinity purified.

2.2. Construction of expression vectors

XK cDNA was a generous gift from Drs. M. Ho and A.P. Monaco, Imperial Cancer Research Fund Laboratories, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, England. Two pRC/CMV expression vectors were constructed. One vector contained only XK cDNA and the other had both XK and Kell cDNAs. For the vector containing only XK, the cDNA was placed in pRc/CMV (Invitrogen, Inc.) in *Hin*dIII and *Xba*I cloning sites. For the vector containing both XK and Kell, the Kell cDNA was modified to contain a Kozak sequence (GCCGCCACC) [26] before the ATG initiation codon. The modified Kell cDNA and XK cDNA were placed in tandem, each with its own CMV promoter and adenylation signal, into pRc/CMV as previously described.

2.3. Transient transfection of COS cells

COS-1 cells (ATCC CRL 1650) were transfected by electroporation using a Gibco Cell Porator with an electrical pulse of 250 V and 330 μF. About 2×10^7 cells were suspended for 15 min in 1 ml of Dulbecco's phosphate-buffered saline with 10 μg of plasmid DNA prior to electroporation. The cells were allowed to recover in ice for 10 min and were seeded at about 5×10^6 cells on 100 mm plastic Petri dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA). The cells were cultured in 10 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum and grown to confluence, usually in 2 days.

2.4. Metabolic labeling of transfected COS cells with L-[^{35}S]methionine

Confluent COS-1 cells were washed with phosphate-buffered saline and incubated at 37°C for 30

min in 1 ml of L-methionine-free Dulbecco's minimal essential medium (Life Technologies, Inc.) containing 0.5 mCi of L-[^{35}S]methionine (NEN Research Products, Boston, MA, USA, specific activity 1110 Ci/mmol). The cells were then washed twice with phosphate-buffered saline and incubated with growth medium for various periods of time.

2.5. Cell fractionation

About 10^8 transfected COS cells were lysed with 2 ml of ice-cold water for 5 min and the cells further disrupted with a loose-fitting Dounce homogenizer. The cell suspension was adjusted to 0.25 M sucrose and centrifuged at $500 \times g$ for 10 min to remove unbroken cells, debris and a nuclear fraction. The supernatant fraction was adjusted to 1.38 M sucrose and overlaid over 2 M sucrose. A discontinuous sucrose gradient of 1.1 M, 0.6 M and 0.25 M sucrose was then applied and centrifuged in a Beckman SW 40.1 rotor at $105\,000 \times g$ for at least 3 h. A rough ER fraction sediments at the 2 M and 1.38 M interface, a smooth ER fraction at the 1.38 M and 1.1 M interface and a Golgi cell fraction at 1.1 M and 0.6 M sucrose [27,28]. The purity of the cell fractions was monitored by electron microscopy (data not shown).

2.6. Surface-labeling of COS cells

Transfected COS cells were surface-labeled with ^{125}I by the lactoperoxidase method [29]. Confluent COS cells, attached to 100 mm Petri dishes, were radiolabeled, washed with phosphate-buffered saline to remove free iodine and solubilized with detergents prior to isolation of XK and Kell/XK complex by immunoprecipitation.

2.7. Immunoprecipitation of Kell/XK complex

COS cells were lysed with 0.5% sodium deoxycholate and 1% dodecyl-β-D-maltoside (Sigma, St. Louis, MO, USA). Included in the lysing solution were the following protease inhibitors: 0.1 mM TPCK, 0.1 mM PMSF and 10 units/ml of aprotinin (Sigma Chem. Co., St. Louis, MO, USA). The lysate was cleared by centrifugation in a microfuge at 12 000 rpm for 10 min at 4°C. Antibodies, to a peptide representing the second loop of XK or to a peptide from the cytoplasmic domain of Kell, were added and incubated overnight at 4°C. The immune complex was isolated with protein A-Sepharose, the proteins eluted with SDS buffer (0.125 M Tris-HCl, pH 6.8, 1% SDS, 5% glycerol) containing 4 M urea and separated on 9% polyacrylamide gels [18,30] or precasted 4–12% Tris-glycine gradient gels (Novex, Sandiego, CA, USA).

The immune complex was treated with endonuclease-H (New England BioLabs Inc., Beverley, MA, USA) to remove the high mannose chitobiose core from glycoproteins [31] as recommended by the manufacturer. The control samples that were not treated with endonuclease-H underwent the same procedure except that the enzyme was not added.

2.8. Two-dimensional gel electrophoresis

In the first dimension, radioactive proteins present in the immunoprecipitate obtained with antibody to XK were separated by SDS-PAGE on a 4–12% Tris-glycine gel (Novex). In the second dimension, an appropriate lane was excised from the gel, soaked in SDS buffer containing 5% 2-mercaptoethanol for 30 min and loaded on top of another precasted 4–12% Tris-glycine gel (Novex).

3. Results

3.1. Time course of Kell/XK assembly

COS cells expressing both Kell and XK proteins were 'pulse'-labeled for 10 or 30 min with L-[^{35}S]methionine. The cells that were pulse-labeled for 10 min were 'chase' incubated for 10, 20, 30, 40 and 50 min and those labeled for 30 min were 'chased' for 1, 2, 3, and 5 h. Radioactive Kell/XK complex was isolated by immunoprecipitation with antibody to XK, the radioactive proteins separated on non-reduced SDS-PAGE, detected by autoradiography and quantitated by densitometry.

At the end of 10 min pulse-labeling with L-[^{35}S]methionine, a prominent radioactive protein was detected with an apparent molecular weight of 38 kDa, the location at which XK migrates (Fig. 1A, lane 1). Another radioactive protein migrating near the top of the gel was also present. The high molec-

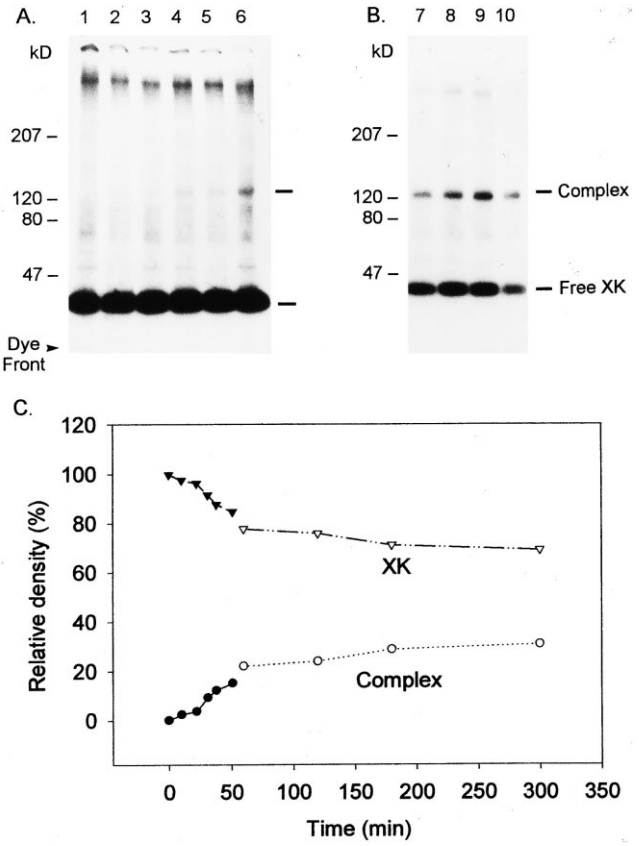

Fig. 1. Time course of Kell/XK assembly. COS cells co-expressing Kell and XK were 'pulse'-labeled either for 10 min and chase incubated at 10 min intervals up to 50 min (panel A) or pulse-labeled for 30 min and chase incubated up to 5 h (panel B). Radioactivity was quantitated from autoradiograms by densitometry and the relative densities, as a percentage of free XK versus Kell/XK complex, at each time point, are graphically presented in panel C. The solid triangles and dots are from panel A and the open triangles and dots from panel B. Panel A: lane 1, 10 min pulse and zero chase; lane 2, 10 min chase; lane 3, 20 min chase; lane 4, 30 min chase; lane 5, 40 min chase; and lane 6, 50 min chase. Panel B: lane 7, 30 min pulse and 1 h chase; lane 8, 2 h chase; lane 9, 3 h chase; and lane 10, 5 h chase.

ular weight protein is also isolated from control COS cells not expressing Kell or XK, suggesting that it is a contaminant co-precipitating during the procedure (data not shown). Although not easily detected at 10 and 20 min, radioactivity was increasingly incorporated into the Kell/XK complex that migrates at 130 kDa during the first 50 min (Fig. 1A). The Kell/XK radioactive complex was not easily apparent until 30 min chase (panel A, lane 4), but densitometry measurements detected increments of incorporation at the earlier times.

The relative radioactivities in free XK and in the Kell/XK complex were quantitated by densitometry and are presented in Fig. 1C. Results from two experiments are shown. In one set COS cells were 'pulsed' for 10 min and 'chased' at 10 min time intervals up to 50 min (Fig. 1A) and in the other set the cells were 'pulsed' for 30 min and 'chased' for 1 to 5 h (Fig. 1B). In the cells pulsed for 10 min there was a linear increase of incorporation of radioactivity into the Kell/XK complex and a concomitant decrease in radioactive 'free' XK. In cells 'pulsed' for 30 min and chased for 1 to 5 h the radioactivity in the Kell/XK complex is near maximal at 1 h of the 'chase' period and there is a slight gradual increase for up to 5 h. Even after 5 h of chase nearly 70% of the radioactivity remained as free XK (Fig. 1B and C).

3.2. *Two-dimensional SDS-PAGE analysis of Kell/XK complex*

COS cells expressing both Kell and XK were metabolically labeled with L-[^{35}S]methionine for 30 min,

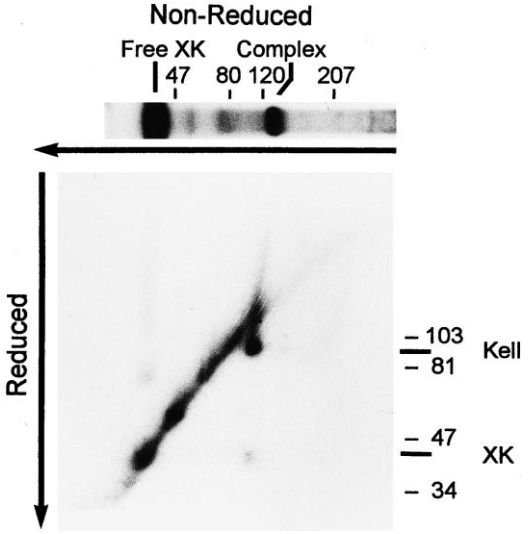

Fig. 2. Two-dimensional SDS-PAGE of Kell/XK complex. COS cells co-expressing Kell and XK were metabolically labeled for 10 min and chase incubated for 3 h. Isolated proteins were first separated under non-reducing conditions (first dimension) then under reducing conditions (second dimension). The locations at which Kell and XK migrate are marked.

'chase' incubated for 3 h and XK and its associated proteins isolated by immunoprecipitation with antibody to XK. The radioactive proteins isolated were analyzed by two-dimensional SDS-PAGE, autoradiography and densitometry. The first dimension was in non-reducing conditions and the second dimension in reducing conditions (Fig. 2). On the first dimension free XK and the Kell/XK complex contained the majority of radioactivity with less amounts in two other proteins with apparent molecular weights of about 60 and 70 kDa. The 60 and 70 kDa proteins appeared more prominent in the second dimension than in the first dimension.

In the second dimension free XK again migrated as a 38 kDa protein, with noticeable trailing of protein radioactivity. The 130 kDa Kell/XK complex, on second dimension, was separated into several radioactive components. The majority of the radioactivity was in Kell (93 kDa) with less amount at 38 kDa, representing free XK. In addition, significant amounts of radioactivity trailed above Kell protein.

The relative radioactivity of Kell and XK components in the complex was determined by densitometry and the values corrected to reflect the methionine contents of these two proteins. Kell has 18 methionine residues and XK has nine. A Kell/XK ratio of 1.34 was obtained.

3.3. *Kell is associated with XK in the endoplasmic reticulum*

COS cells expressing both XK and Kell were labeled for 1 h with L-[^{35}S]methionine and some were 'chase' incubated for 5 h. The cells labeled for 1 h were fractionated into rough and smooth endoplasmic reticulum cell fractions. A Golgi cell fraction was obtained from cells 'chased' for 5 h. Kell/XK complex was isolated from each of the fractions with antibody to XK, treated with endonuclease-H and separated by SDS-PAGE in reducing conditions (Fig. 3). Endonuclease-H removes N-linked mannose-rich oligosaccharides from glycoproteins [31] present in the endoplasmic reticulum (ER) and does not cleave oligosaccharides from glycoproteins whose N-linked sugars have been further processed in the Golgi cell fractions [32]. Kell, but not XK, is a glycoprotein, and therefore endonuclease-H treatment provides information on oligosaccharide processing and cellular location of the Kell component of the Kell/XK complex.

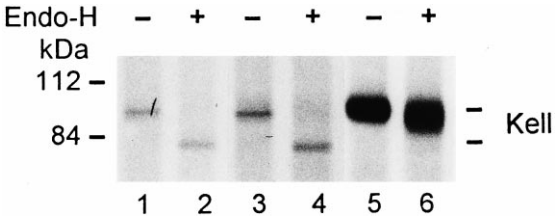

Fig. 3. Kell/XK complex in the endoplasmic reticulum and Golgi cell fractions. COS cells co-expressing Kell and XK were metabolically labeled for 1 h and chase incubated for 5 h. Rough and smooth ER fractions were obtained at 1 h of incubation and Golgi cell fractions at the 5 h chase period. Kell/XK complex was isolated with antibody to XK, treated with endonuclease-H and separated on reduced SDS-PAGE. Lanes 1 and 2 are from rough ER samples; lanes 3 and 4 from smooth ER; and lanes 5 and 6 from Golgi cell fractions. Lanes 1, 3 and 5 were untreated and lanes 2, 4 and 6 were treated with endonuclease-H.

In both the rough and smooth endoplasmic reticulum fractions, antibody to XK co-isolated radioactive Kell protein. The Kell component of the Kell/XK complex was cleaved by endonuclease-H, migrating faster on SDS-PAGE, and indicating that, as expected from an N-linked glycoprotein present in the ER, Kell, at this stage, contains high mannose sugars (Fig. 3, lanes 1–4).

A Kell/XK complex, evidenced by the co-isolation of Kell with antibody to XK, was also obtained from the Golgi cell fraction, measured after 5 h of chase incubation. As compared to Kell present in the ER fractions, the Kell component in the Golgi was endonuclease-H insensitive, indicating processing of its N-linked sugars (Fig. 3, lanes 5 and 6).

These results demonstrate that Kell/XK linkage commences in the ER and then proceeds to the Golgi cell fraction.

3.4. *Endonuclease-H insensitivity of Kell from red cell membranes*

Kell, expressed in COS cells, migrates slightly faster on SDS-PAGE when treated with endonuclease-H, even after it has entered the Golgi compartment (Fig. 3, lanes 5 and 6). The slight sensitivity of Kell to endonuclease-H at times when oligosaccharide processing has occurred may be due to several reasons. It could be due to aberrant N-glycosylation of

Kell by COS cells, to a proteolytic contaminant in the endonuclease-H or could be a normal feature of Kell glycoproteins. To distinguish between these possibilities, Kell protein, isolated from normal human red cells, was treated with endonuclease-H, separated by reduced SDS-PAGE and identified by Western immunoblotting (Fig. 4). Endonuclease-H treatment did not affect the electrophoretic mobility of Kell isolated from red cells, indicating that the endonuclease-H did not contain proteolytic enzymes and that the electrophoretic mobility of Kell from red cells is not affected by endonuclease-H. Therefore these results suggest that recombinant Kell, expressed by COS cells, may have different N-linked sugars than Kell present in red cells and this results in a slightly faster electrophoretic migration on treatment with endonuclease-H.

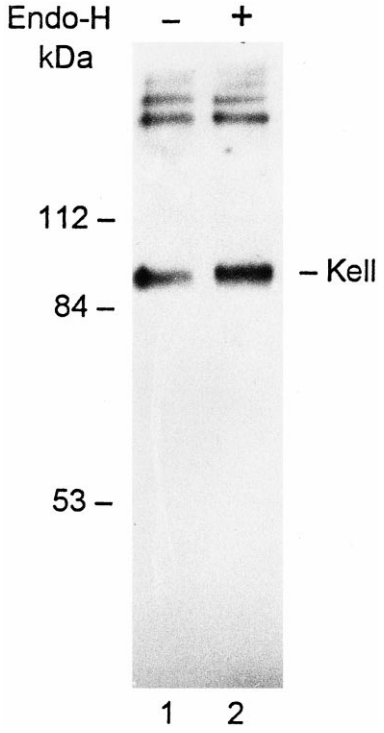

Fig. 4. Kell from human red cells is not cleaved by endonuclease-H. Kell protein, isolated from red cells, was treated with endonuclease-H separated by reduced SDS-PAGE and detected by Western immunoblotting using antibody to Kell. Lane 1 contains the untreated sample and lane 2 the endonuclease-H-treated sample.

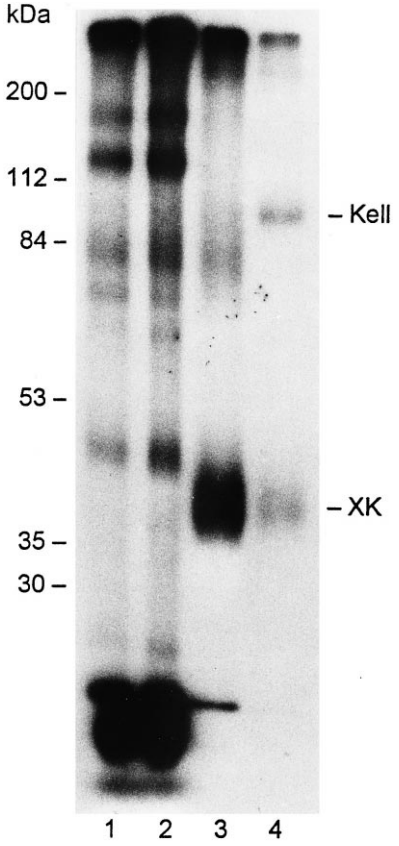

Fig. 5. Kell and XK are surface-exposed. COS cells expressing XK alone or both Kell and XK were surface-labeled with ^{125}I by the lactoperoxidase-catalyzed method. The cells were lysed and XK, or Kell/XK complexes, isolated with antibody to XK. Proteins were separated by reduced SDS-PAGE and detected by autoradiography. Lane 1 shows total surface-labeled proteins from COS cells expressing only XK and lane 2 total surface-labeled proteins from cells co-expressing Kell and XK. Lanes 3 and 4 show surface-exposed proteins that were immunoprecipitated with antibody to XK. Lane 3 shows radioactive proteins from cells only expressing XK and lane 4 from cells co-expressing both Kell and XK. The locations at which Kell and XK migrate are marked.

3.5. *XK can be transported to the cell surface in the absence of Kell*

Previous studies in which Kell was expressed in transfected cells demonstrated that Kell protein travels to the cell surface and expresses Kell blood group antigens [15,19]. To determine whether XK can also be transported to the cell surface in the absence of Kell, COS cells expressing only XK, or both Kell and XK, were surface-labeled by the lactoperoxidase method with radioactive iodine. The washed cells

were lysed with detergent and XK isolated with antibody to XK. Separation of the total cellular proteins on reduced SDS-PAGE, from both the COS cells expressing only XK, or expressing both Kell and XK, showed a simple pattern of radioactive proteins indicating that, as expected from the lactoperoxidase method, only surface-exposed proteins were labeled (Fig. 5, lanes 1 and 2). There was little, or no, difference in the pattern from cells expressing only XK or expressing both Kell and XK indicating that Kell and XK represent a minor fraction of surface-exposed protein. Antibody to XK, however, isolated surface-iodinated XK from COS cells expressing only XK demonstrating that XK is present on the cell surface (Fig. 5, lane 3). In COS cells co-expressing both Kell and XK, antibody to XK isolated both radioactive XK and Kell indicating that the complex was also present on the cell surface (Fig. 5, lane 4). Less XK was placed on the cell surface when both Kell and XK were co-expressed than when XK was expressed by itself (Fig. 5, lanes 3 and 4).

4. Discussion

Both Kell and XK are integral membrane proteins that are partially exposed on the red cell surface. Their structures are very different; Kell is a glycoprotein that spans the membrane once [1] and XK is not glycosylated and traverses the membrane 10 times with both the N- and C-termini in the cytoplasm [2]. On red cells, Kell and XK are linked by an extracellular disulfide bond [3,4] connected by Kell Cys^{72} and XK Cys^{347} [18]. Our studies demonstrate that in transfected COS cells, Kell and XK are first linked in the ER and are transported, via the normal secretory pathway, to the Golgi and the plasma membrane.

Previous studies established that Kell and XK form a disulfide-linked complex that migrates on SDS-PAGE with an apparent molecular weight of approximately 130 kDa. The two proteins are linked by a single disulfide bond between Kell Cys^{72} and XK Cys^{347}, since substitution of these cysteine residues with serine abolished the formation of the 130 kDa Kell/XK complex [18]. In the present studies we confirm, using antibody to XK rather than antibody to Kell and by two-dimensional SDS-PAGE analysis, that Kell and XK are the major components of the 130 kDa complex. The molar ratios of Kell and XK, as determined by densitometry and correction of the radioactive values to reflect different methionine content, indicate a Kell/XK ratio of 1.34. The lower amount of XK may be due to a lower recovery of XK from the complex due to aggregation. XK is a very hydrophobic protein that often migrates on SDS-PAGE, even in reducing conditions, as an aggregate. As noted in Fig. 2, the XK that migrated as 'free' XK on the first dimension produced some trailing or streak in the second dimension. The XK component of the Kell/XK complex behaves similarly and the aggregated form is not measured, leading to low recoveries. Aggregation of XK has also been described by others [4].

The environment of the ER encourages protein assembly and disulfide linkages, since it maintains an oxidative redox potential, contains protein disulfide isomerase which catalyzes disulfide formation, and also has other resident chaperone proteins that facilitate folding and assembly of protein complexes [33–35]. As noted in Fig. 1, after a 10 min 'pulse' incubation some complex formation was measured 10 min into the 'chase' period and the disulfide linkage of Kell and XK proceeds linearly for about an hour. Multi-component membrane complexes, such as the T cell antigen receptor, which is composed of at least seven polypeptides, some of which are disulfide-linked, show different rates of assembly for their component parts. Similarly to the assembly of Kell and XK the disulfide linkage of α and β component polypeptides of T cell antigen receptor also occurs slowly, with an estimated half time of 10 to 30 min [36,37].

Two lines of evidence show that Kell and XK are disulfide-linked in the ER. The first and most direct evidence is that cell fractionation studies showed that the Kell/XK complex was present in both the rough and the smooth ER cell fractions. Secondly, the Kell component of Kell/XK was cleaved by endonuclease-H, confirming a pre-Golgi cellular location (Fig. 3).

Kell can travel to the cell surface and appears to be properly folded in the absence of XK. Previous studies showed that, in the absence of XK, Kell is surface-expressed and acquires a tertiary structure that resembles the native protein, since it exhibits conformational antigenic epitopes [15,19]. In this

study we demonstrate that XK can also be transported to the cell surface in the absence of Kell and that when co-expressed both Kell and XK exist as a disulfide-bonded complex on the plasma membrane (Fig. 5).

The recombinant expression of Kell and XK by COS cells does not completely mirror the in vivo situation. Two rare red cell phenotypes, involving Kell and XK, suggest that co-expression of Kell and XK is necessary for optimal expression of both proteins on the cell surface. In the McLeod phenotype, red cells lack XK and there is an accompanying marked decrease in the amount of Kell present on the membrane. In the Ko (null) phenotype the red cells have no detectable Kell protein and have decreased amounts of XK protein, although as measured by serological procedures the Ko (null) red cells have enhanced amounts of an antigen, Kx, that is carried on XK protein. In the recombinant system, co-expression of Kell and XK does not enhance the amount of Kell or XK that is placed on the cell surface.

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