The Kell blood group protein is a metalloendopeptidase that preferentially cleaves a Trp\(^{21}\)-Ile\(^{22}\) bond of big endothelin-3 producing bioactive endothelin-3. Kell is a polymorphic protein, and 25 different phenotypes, because of point mutations resulting in single amino acid substitutions, have been described. It was recently reported that a recombinant form of KEL1 (K, K1) phenotype, expressed in K562 and HEK293 cells, had no endothelin-3-converting activity, in contrast to the common KEL2 (k, K2) phenotype. We demonstrate that KEL1 red blood cells and also a soluble recombinant form of KEL1 protein (s-Kell KEL1) have similar enzymatic activity as the common Kell phenotype. In addition we show that KEL6 red blood cells, which are more prevalent in persons of African heritage than in Caucasians also have endothelin-3-converting enzyme activity and that the recombinant soluble form of KEL6 protein (s-Kell KEL6) has similar \(K_m\) values as the wild-type.

The Kell blood group system is highly polymorphic, expressing over 25 antigens that have been classified into five antithetical sets of high and low prevalence antigens with the others being independently expressed or having unknown antithetical partners. The different phenotypes are due to single nucleotide mutations that result in an amino acid substitution (1, 2). Two antithetical allelic sets of antigens, KEL1 (K, K1)/KEL2 (k, K2) and KEL6 (Js)/KEL7 (Js\(^{\#}\)) are of particular interest because they are more prevalent in certain racial groups. For example, KEL1 is present in 9% of Caucasians and in 2% of persons of African heritage, whereas KEL6 is present in 20% of persons of African heritage and in less than 1% of Caucasians. KEL1 is due to a 698C\(\rightarrow\)T mutation that results in a Thr\(^{193}\)\(\rightarrow\)Met substitution at an amino acid residue whose substitutions lead to the different phenotypes. In some cases, for example X-converting enzyme, the substrates are unknown. Unlike endothelin-converting enzyme-1 and -2 that preferentially activate endothelin-1 (ET\(^{1}\)) (10, 11), Kell has strong preference for big endothelin-3 (big ET-3) and has much less activity with big endothelin-1 (big ET-1) and big endothelin-2 (big ET-2) (15). Within the M13 family, Kell is unique in that it is linked, through a single disulfide bond (16, 17), with another protein, XK, a 50.9-kDa putative membrane transporter with 10 possible transmembrane domains (18). However, the extracellular domain of Kell expressed as a soluble recombinant form retains its endothelin-3-converting enzyme activity. A three-dimensional model of Kell protein, constructed on the basis of the crystal structure of neutral endopeptidase 24.11, showed that Kell has two globular extracellular domains (19). The domain closest to the cell membrane contains the enzyme-active site. The surface of the other domain, away from the enzyme active site contains the amino acid residues whose substitutions lead to the different phenotypes.

Because of the unequal distribution of KEL1 and KEL6 in different racial groups we studied their catalytic activity. In contrast to the recent report by Clapéron et al. (20), we demonstrate that KEL1 red blood cells and a recombinant form of KEL1 phenotype have a similar big ET-3-cleaving activity to the common KEL2 phenotype.

**MATERIALS AND METHODS**

*Construction of Recombinant Soluble KEL1 Protein (s-Kell KEL1)*—Two overlapping PCR products were produced using each one of the primers containing the KEL1 mutation. The template for the PCR was the common Kell cDNA. The two overlapping PCR products were joined by another PCR to yield the complete cDNA. The primer sequences of the two PCRs are as follows: PCR1, (EX1F) 5’-CAGTCC-TCCGAATCAGCTCCTAGA-3’ and (K1R) 5’-ACTGACCTCATCAGA-GTCTCAGCATTCCG-3’; PCR2, (NK1F) 5’-GGACTTCTTTAAA-ACCTTTACCGAATGCTG-3’ and (2316R) 5’-GCTGTGGCATCCTT-GTGTTACC-3’. The underlined bold letter in the primer sequence is the incorporated KEL1 mutation.

The 230-bp cDNA segment with the KEL1 mutation, obtained by cutting the PCR product with EcoRI and AvrII, replaced a DNA segment between EcoRI and AvrII sites of a wild-type s-Kell construct with an N-terminal six-histidine tag, which was in a pAcGp67A vector (BD Biosciences) (15, 20). The sequence of the construct was verified by DNA sequencing. The preparation of common s-Kell and s-Kell6 expression constructs has been described previously (15).

*Expression of Recombinant Proteins*—Recombinant s-Kell proteins were expressed in serum-free condition using Ex-Cell 400 medium (IRH Biosciences, St. Lenexa, KS) and sf9 cells (Invitrogen) following the...
manufacturer’s protocol. The absence of cross-contamination in s-Kell KEL1 with common s-Kell was determined by isolation of the baculovirus DNA from the expression culture and sequencing the KEL1 region that was amplified by PCR.

Isolation of Recombinant s-Kell Proteins—The secreted s-Kell proteins were harvested by affinity chromatography on nickel columns basically following the procedure described previously (15). s-Kell in the cell medium (3 x 10⁶ cells/ml) was applied to a nickel-nitrilotriacetic acid resin column (Ni-NTA spin kit, Qiagen, Valencia, CA) that had been equilibrated with serum-free cell culture medium (Ex-cell 400 or SF-900 II from Invitrogen). The column was washed once with 50 mmol/liter HEPES buffer, pH 7.4, containing 0.3 mol/liter NaCl and 20 mmol/liter imidazole and once with the same buffer but with imidazole increased to 50 mmol/liter. Bound protein was eluted with 50 mmol/liter HEPES, pH 7.4, containing 0.3 mol/liter NaCl, and 250 mmol/liter imidazole. The above solutions were made in the serum-free media instead of H₂O. The serum-free medium contains non-ionic detergent, which is necessary to maintain the Kell enzyme activity. If the solutions are not made with the serum-free medium, 0.1% Tween 20 is required to retain enzyme activity.

Endothelin-converting Enzyme Analysis of KEL1 and KEL6 and Common Kell Phenotype—The recombinant Kell proteins and the human red cells of KEL1, KEL6, Kell null, and common Kell phenotype were assayed for their abilities to process big ET-1, big ET-2, and big ET-3 using an endothelin enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI) that was previously described (15). Big ET-1, big ET-2, or big ET-3 (0.1 μmol/liter) were incubated at 37 °C for 10 min with assay buffer (50 mmol/liter HEPES, pH 6.0, 50 μmol/liter ZnCl₂, and 150 mmol/liter NaCl containing 0.25% (v/v) of supernatant of boiled bovine serum albumin (1 mg/ml)) and various amounts of s-Kell. The final volume was 200 μl. At the end of the incubation time, an equal volume of 5 mmol/liter EDTA was added to terminate the reaction, and the samples were kept in ice until enzyme immunoassay was performed. To determine whether the KEL1 protein is inactivated by the conditions used by Clapeçon et al. (20), the purified s-Kell and s-Kell KEL1 were stored at 4 °C for 16 h in the presence of 2% Brij-58 (Sigma-Aldrich) and 1% Triton X-100 (Sigma) prior to the enzyme assay. When Km values were determined for big ET-3, the substrate concentrations ranged from 0.1 to 0.6 μmol/liter, and the incubation time was 10 min.

RESULTS

KEL1 and KEL6 Phenotype Red Blood Cells Have Endothelin-3-converting Enzyme Activities Comparable to Common Kell-type Red Blood Cells—The endothelin-converting enzyme activities of red blood cells with KEL1, Kell null and common phenotype were compared. As previously shown, common Kell-type red blood cells preferentially cleave big ET-3 releasing ET-3 and have much less activity with big ET-1 and
Endothelin-3-converting Activities of KEL1 and KEL6 Phenotypes

TABLE 1  

| s-Kell phenotype | $K_m$ ± S.D. (μM) | $k_{cat}$ ± S.D. (s$^{-1}$) |
|------------------|------------------|---------------------|
| s-Kell, common   | 1.05 +/− 0.54    | 0.27 +/− 0.14       |
| s-Kell KEL1 (K1) | 1.39 +/− 0.32    | 0.24 +/− 0.07       |
| s-Kell KEL6 (Je) | 1.06 +/− 0.33    | 0.20 +/− 0.08       |

big ET-2 as substrates. Kell null red blood cells have minimal background activity (15, 20). The KEL1 red blood cells were comparable to the common Kell red blood cells and also preferentially activated ET-3 (Fig. 1A). The KEL6 (Je) red blood cells, like the common Kell type red blood cells, were also capable of cleaving big ET-3 and producing ET-3 (data not shown).

Soluble Recombinant Proteins of Common Kell-type, KEL1 Phenotype, and KEL6 Phenotype Have the Same Endothelin-3-converting Enzyme Activities—$K_m$ and $k_{cat}$ values for common Kell, KEL1, and KEL6 soluble recombinant proteins, using big ET-3 as substrate, were measured by enzyme immunoassay. The results are summarized in Table 1. KEL1 and KEL6 proteins had similar $K_m$ and $k_{cat}$ values as the common Kell protein. In addition, s-Kell KEL1 and common s-Kell both retained enzyme activities when stored at 4°C for 16 h in the presence of 2% Brij-58 and 1% Triton X-100.

DISCUSSION

In contrast to a recent publication by Clapéron et al. (20), which reported that recombinant KELL protein expressed on the surface of HEK293 and K562 cells did not have endothelin-3-converting enzyme activity, whereas the common KELL2 did, our results clearly demonstrated that both KELL1 red blood cells and a soluble recombinant form of KELL1 have similar enzyme activity as the wild-type forms. The reasons for the discrepancy are not clear. The possibility of loss of enzyme activity of KELL1 protein during purification of the recombinant native KELL1 (K1) was checked by storing the purified wild-type s-Kell and s-Kell KEL1 in the same conditions as described by Clapéron et al. (20). The soluble recombinant proteins were kept in the presence of 2% Brij-58 and 1% Triton X-100 at 4°C for 16 h prior to the enzyme assay. There was no reduction in s-Kell KELL1 enzyme activity.

The KELL1 phenotype arises from a Thr$^{193}$ → Met substitution that occurs in an N-glycosylation site, and KELL1 is expected to have four instead of five N-linked sugar moieties. It is possible that this mutation may affect the processing and insertion of KELL protein on the cell surface in a recombinant system. This is unlikely, however, because the KELL1 recombinant protein was detected by antibodies on the cell surface.

In our study both intact KELL1 and KELL6 red blood cells, which mimic the in vivo conditions, and soluble recombinant proteins that express the extracellular domains show very similar activity to their wild-type counterparts. This is perhaps not surprising because a three-dimensional model of the extracellular sequences of KELL protein indicates the presence of two separate globular domains. The mutation that leads to the KELL1 amino acid substitution is present in the outer globular domain, quite distinct from the globular domain that is closer to the plasma membrane and contains the enzyme active site. Although the

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