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Molecular cloning and primary structure of Kell blood group protein

(cDNA/neutral endopeptidase)

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ABSTRACT **The Kell blood group is a major antigenic system in human erythrocytes. Kell antigens reside on a 93-kDa membrane glycoprotein that is surface-exposed and associated with the underlying cytoskeleton. We isolated tryptic peptides and, based on the amino acid sequence of one of the peptides and by using the PCR, prepared a specific oligonucleotide to screen a λgt10 human bone-marrow cDNA library. Four clones were isolated, one containing cDNA with an open reading frame for an 83-kDa protein. All known Kell amino acid sequences were present in the deduced sequence; moreover, rabbit antibody to a 30-amino acid peptide, prepared from this sequence, reacted on an immunoblot with authentic Kell protein. The Kell cDNA sequence predicts a 732-amino acid protein. Hydropathy analysis indicates a single membrane-spanning region, suggesting that Kell protein is oriented with 47 of its N-terminal amino acids in the cell cytoplasm, and a 665-amino acid segment, which contains six possible N-glycosylation sites, is located extracellularly. Computer-based search showed that Kell has structural and sequence homology to a family of zinc metalloglycoproteins with neutral endopeptidase activity.**

The Kell blood group system is important in transfusion medicine. It is a complex system containing at least 24 antigens (1, 2) residing on a 93,000-kDa glycoprotein that is surface-exposed, spans the erythrocyte membrane, and is attached to the underlying cytoskeleton (3, 4). Kell glycoproteins contain ≈12% carbohydrate by weight, all of which are N-linked glycosides (5, 6).

A null phenotype (called Ko) lacks all known Kell antigens but has enhanced expression of a related antigen, named Kx. Kx is present in all erythrocytes, except those of the rare McLeod type; McLeod erythrocytes have weakened Kell antigens and acanthocytic morphology (1, 2). Kx antigen is carried on a 37-kDa protein (7). McLeod individuals also exhibit neurological and muscular abnormalities (1, 2). Kell and McLeod are coded by different genes: The 93-kDa Kell protein is derived from an autosomal gene, which has not yet been mapped, and the McLeod gene is X chromosome-linked and located at Xp21 (8).

We have isolated and partially characterized the protein that carries Kell antigens (4). However, the complete primary structure and possible function of this membrane protein have not yet been determined. To define its structure we have cloned Kell cDNA, deduced its amino acid sequence, and noted that Kell protein has structural and sequence homology with a family of zinc-binding neutral endopeptidases.†

MATERIALS AND METHODS

Protein Purification and Microsequencing of Tryptic Peptides. Kell protein was isolated, as described (3, 4), by immunoprecipitation from intact erythrocytes, with a murine monoclonal antibody to K14 and by SDS/PAGE under reducing conditions. The 93-kDa protein was further purified by reverse-phase HPLC on a C_4 column (Vydac) with a linear gradient of acetonitrile (0–100%) in 0.01% trifluoroacetic acid. HPLC-purified Kell protein (50–75 μg) was treated with two additions of *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Millipore) and incubated overnight at 37°C to ensure complete digestion. The digested peptides were separated by HPLC on a butyldimethylsilyl-bonded phase guard column (Supelco) with a linear gradient (0–100%) of acetonitrile in 0.01% trifluoroacetic acid. Three of the peptides, appearing as major peaks, were further purified by HPLC with two different chromatographic systems. These procedures have been illustrated (9). The purified peptides were sequenced by using an Applied Biosystems (model 477A) protein sequencer.

The following unequivocal amino acid sequences were obtained: peptide 1, Leu-Phe-Gln-Met-Val-Thr-Ile-Asp-Gln-Leu-Lys; peptide 2, Gly-Glu-Thr-Val-Leu-Pro-Ser-Leu-Asp-Leu-Ser-Pro-Gln-Gln-Ile-Phe-Phe-Arg; peptide 3, His-Gly-Pro-Leu-Ser-Ser-Thr-Pro-Ala-Phe-Ala-Arg.

Preparation of Kell-Specific Probe to Screen a Human Bone-Marrow cDNA Library. To obtain a stringent oligonucleotide probe the following primers, deduced from the amino acid sequence of peptide 2, were synthesized. The sense primer (5′-GGN GAR ACN GTN PTN-3′) coded for amino acids Gly-Gln-Thr-Val-Leu, and the antisense primer (3′-GTY GTY TAP AAR AAR-5′) coded for Gln-Gln-Ile-Phe-Phe. In the above primers, N = A, T, G, or C; R = G or A; Y = T or C; and P = T, G, or A. An aliquot [representing ≈5 × 10^5 plaque-forming units (pfu)] was taken from a λgt10 human bone-marrow cDNA library (Clontech), and DNA was prepared for use as template (10). In some cases, human genomic DNA was also used as a template with similar results. The PCR was done with the GeneAmp DNA amplification reagent kit from Perkin–Elmer/Cetus with slight modifications. Concentrations of template, primers, and magnesium were increased (2–10 μg of DNA template, 0.5 nmol of primer per 0.1 ml of reaction mixture, and the final $MgCl_2$ concentration was 5 mM). The annealing temperature was 37°C, and 25 cycles were used. Several DNAs of multiple sizes were amplified. However, on a Southern blot with a mixed oligonucleotide probe deduced from an internal amino acid sequence (Pro-Ser-Leu-Asp-Leu-Ser-Pro) of peptide 2, hybridization occurred only at the predicted size of 51 base pairs (bp). This 51-bp DNA was subcloned by blunt-end ligation to pUC18 (BRL) for sequencing. The following sequence was obtained: 5′-GGA GAG ACA GTG CTG CCC AGC CTG GAC CTC AGC CCC CAG CAG ATC TTC

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Abbreviations: CALLA, common acute lymphoblastic leukemia antigen; pfu, plaque-forming units.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M64934).

TTC-3'. This base sequence corresponds to 17 amino acids in peptide 2.

Screening of a Human Bone-Marrow cDNA Library. A λgt10 human bone-marrow cDNA library purchased from Clontech was screened with the 51-mer probe described above, labeled at its 5' end with ^{32}P. About 1.2×10^6 plaques were analyzed (10).

Isolation and Sequencing of cDNA Inserts. Phage DNA was prepared by using Lambdasorb phage adsorbent (Promega). The DNA inserts were released with *Eco*RI and subcloned into pGEM-7Zf(+) (Promega). DNA sequencing was done by two methods. (*i*) The 1.9-kb insert from clone 191 was sequenced by Lark Sequencing Technologies (Houston) (11, 12). (*ii*) DNA sequencing was done on an automatic Genesis 2000 DNA sequencer. The complementary chains of DNA, synthesized by Sequenase, were terminated by fluorescence-tagged dideoxynucleotide triphosphates (13). Both strands were sequenced.

PCR to Determine Flanking Regions of the Open Reading Frame. One clone, 191, contained an insert that, when treated with *Eco*RI, yielded three fragments—0.5, 1.9, and 3 kilobases (kb) in size. The 1.9-kb fragment hybridized with the 51-mer Kell-specific oligonucleotide probe (see Fig. 1). To determine the flanking relationships of the 0.5- and 3-kb fragments to the 1.9-kb fragment, PCR were performed with primers from either the 3' or the 5' end of the 1.9-kb fragment and forward and reverse primers from λgt10 (Promega). From the 5' end an antisense primer, bases 569–590 (Fig. 2) was used, and from the 3' end, the base sequence was 2403–2422 (Fig. 2). DNA isolated from clone 191 was used as template. In these experiments the conditions recommended by Perkin–Elmer/Cetus for the GeneAmp kit were used, except that the annealing temperature was 44°C.

Northern (RNA) Blot Analysis. Human bone marrow was stored in 5 vol (wt/vol) of 6 M guanidine thiocyanate/12.5 mM sodium citrate, pH 7.0/5% 2-mercaptoethanol and kept frozen at −70°C. Total RNA was isolated by CsCl-gradient centrifugation (10) and further purified by the acid guanidine thiocyanate–phenol/chloroform extraction procedure (14). Poly(A)$^+$ RNA was isolated by using an mRNA isolation kit purchased from Invitrogen. The probe used was a random-labeled 1.9-kb fragment from clone 191. The analysis was done as described by Sambrook *et al.* (10).

Preparation of Antibody and Immunoblots. A peptide, corresponding to the 30 C-terminal amino acids in the open reading frame of Kell cDNA, was synthesized. Rabbits were immunized by injections of 250 μg of the peptide in Freund's adjuvant. Immunoblot analysis was done with 200 times diluted antiserum. Kell protein was isolated by immunoprecipitation from common Kell erythrocytes with human antibody to K7 (3, 4). Total erythrocyte-membrane proteins were prepared from erythrocytes of Kell-positive and Ko individuals. Either ^{125}I-labeled goat anti-rabbit IgG F(ab')$_2$ (New England Nuclear) or horseradish peroxidase-conjugated swine anti-rabbit IgG (Accurate Chemicals, Westbury, NY) was used as second antibody.

General Methods. Standard molecular biology procedures were used for DNA and RNA isolation, screening of the cDNA library, subcloning, and Northern, Southern, and immunoblots (10).

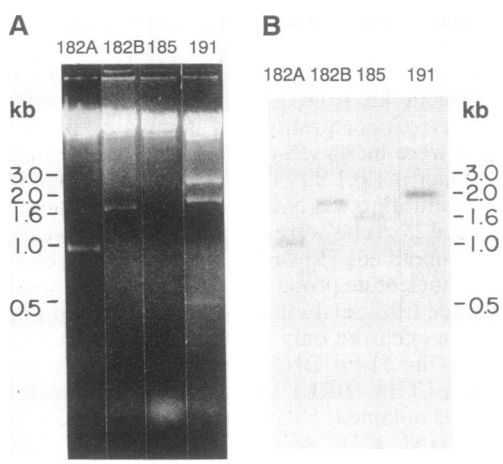

FIG. 1. Kell cDNA clones. Four clones that hybridized with a Kell-specific 51-mer oligonucleotide were isolated from a λgt10 human bone-marrow cDNA library. (*A*) DNA inserts released with *Eco*RI. (*B*) Southern blots with the 51-mer oligonucleotide as a probe.

RESULTS AND DISCUSSION

Isolation and Characterization of Kell cDNA. The strategy for cloning Kell cDNA was to prepare an oligonucleotide probe that would exactly match base sequences in the library to be screened, allowing us to use stringent screening conditions that would minimize false positives. With this aim PCR technology was applied, using the amino acid sequence of one of the Kell tryptic peptides as a starting point. Mixed oligonucleotides corresponding to the N- and C-terminal segments of peptide 2 were used as primers, and an aliquot of DNA was prepared from the λgt10 human bone-marrow cDNA library or genomic DNA for template. PCR yielded a 51-mer oligonucleotide that corresponded to the amino acid sequence of peptide 2. When Kell cDNA was sequenced (Fig. 2), there was a 4-nucleotide mismatch from the redundant primers used in the PCR. The PCR result, however, both yielded a specific oligonucleotide probe with which to screen the library and also established that the partial Kell sequence was present in the library. Of 1.2×10^6 plaques screened with this oligonucleotide, four positive clones were obtained. One of the clones (191), when treated with *Eco*RI, produced three inserts: 0.5 kb, 1.9 kb, and 3 kb. Only the 1.9-kb fragment hybridized with the 51-mer PCR oligonucleotide used in screening (Fig. 1). The 1.9-kb fragment of clone 191 was the largest fragment that hybridized with our specific probe and was sequenced first. Bases 531–2458 (Fig. 2) are the sequences obtained from the 1.9-kb fragment of clone 191. The 5' end contained an *Eco*RI site, and at position 2453 (Fig. 2) there was a typical polyadenylylation signal. A termination codon (TAA) appeared at position 2320 (Fig. 2). A large open reading frame started at the 5' *Eco*RI site and continued uninterrupted to the termination codon. Taken together, these results suggested that the 1.9-kb fragment had the complete 3' end of Kell cDNA but was missing portions of the 5' end because this fragment was not large enough to code for the entire Kell protein. The presence of two other (0.5 and 3 kb) fragments in the same clone suggested a long transcript with internal *Eco*RI sites.

To determine orientation of the 0.5- and 3-kb fragments with regard to the 1.9-kb fragment, PCR was performed by using forward and reverse λgt10 primers and oligonucleotides from the 3' and 5' end of the 1.9-kb fragment. DNA from clone 191 was used as a template. When a forward λgt10 primer and an antisense primer from the 5' end of the 1.9-kb fragment (base positions 578–590 in Fig. 2) were used, a 600-bp product was obtained. The 600-bp PCR product should contain the λgt10 primer at its 5' end, followed by a 500-bp section identical to the 0.5-kb fragment in clone 191; at its 3' end it should overlap with bases 531–590 (Fig. 2), which are at the 5' end of the 1.9-kb fragment. This was the case. The 600-bp PCR product hybridized with the 0.5-kb fragment in clone 191 (data not shown) and, when sequenced, its 3' end had complete base identity with the expected 59-base overlap at the 5' end of the 1.9-kb fragment (positions

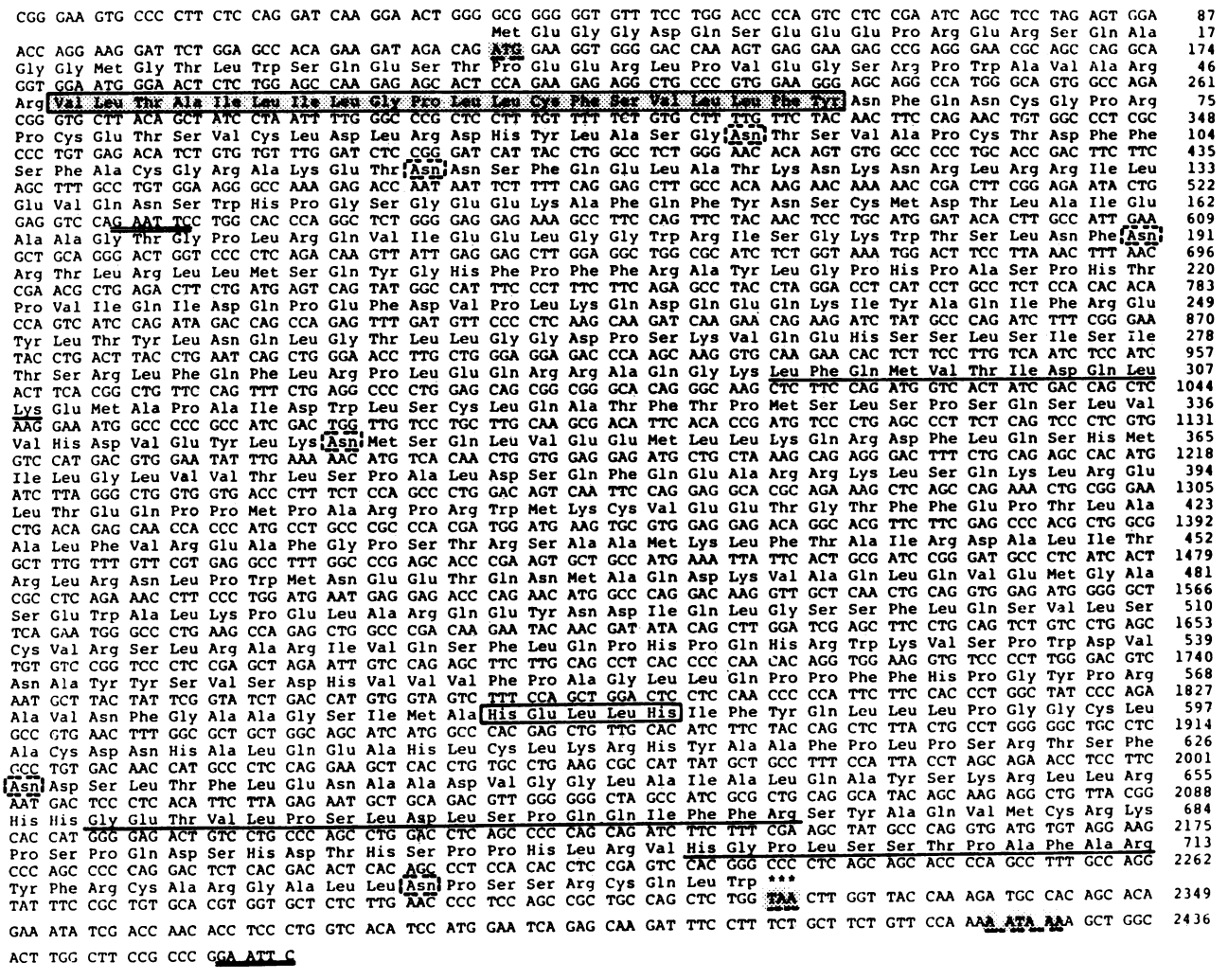

FIG. 2. Nucleotide and deduced amino acid sequences of Kell cDNA. The putative membrane-spanning region is shown in a shaded box; the three regions underlined by solid lines show amino acid sequences of Kell tryptic peptides; six possible N-linked glycosylation sites are indicated by boxed asparagine residues; the initiation codon (ATG), a termination codon (TAA), and a polyadenylylation signal (AATAAA) are underlined by broken lines; two *Eco*RI sites are underlined by double lines; and the pentapeptide consensus sequence, which is part of the zinc-binding domain of neutral endopeptidases, is shown in a clear box.

531–590 in Fig. 2). This result demonstrated that the 0.5-kb and 1.9-kb fragments are contiguous and that the 0.5-kb fragment is situated at the 5′ end of the 1.9-kb fragment. The complete base sequence of the 600-bp PCR product and the 1.9-kb fragment encompasses an open reading frame coding for an 83-kDa protein; the complete sequence is given in Fig. 2. The three known Kell amino acid sequences are represented in the open reading frame, demonstrating that Kell cDNA was isolated (Fig. 2).

When a reverse λgt10 primer and a sense primer from the 3′ end of the 1.9-kb fragment (positions 2403–2422) were used in a PCR reaction, a 3-kb product was obtained that hybridized with the 3-kb fragment from clone 191. This result indicated that the 3-kb fragment was at the 3′ end of the 1.9-kb fragment. The 3-kb fragment has not yet been sequenced, but because it follows a termination codon and a polyadenylylation signal, it probably represents a large 3′ untranslated region.

RNA Analysis of Bone Marrow. Northern blot analysis of human bone-marrow mRNA with radiolabeled 1.9-kb fragment from clone 191 gave a weak, but positive, hybridization signal at 2.6 kb (Fig. 3). This result indicates a smaller transcript than predicted by the cDNA in clone 191. Restriction fragment analysis demonstrated that cDNAs in clones 182A, 182B, and 185 have smaller 3′ untranslated regions than clone 191 (data not shown), in accordance with a 2.6-kb transcript.

Structure of Kell Protein. Kell protein has a blocked N terminus (6, 9); therefore, pyroglutamic acid is probably the N-terminal residue. Glutamic acid, which is easily deaminated to form pyroglutamic acid, follows the initiation methionine. Posttranslational cleavage of N-terminal methionine has been described in other membrane-associated proteins (15). Because we could not determine the N terminus of Kell protein by amino acid sequencing of the intact protein, we base our current assessment on the fact that a termination (TAG) codon (base 79, Fig. 2) precedes initiation codon ATG (base 125, Fig. 2). The ATG codon starts an open reading frame that codes for 732 amino acid with a M_r of 82,793. We had previously determined, based on SDS/PAGE mobility, that deglycosylated Kell protein is ≈79 kDa (5).

Hydropathy analysis (16) of the predicted amino acid sequences of Kell protein indicates that it contains one possible membrane-spanning domain (amino acids 48–67). The N-terminal 47 amino acids, which precede the membrane-spanning region, are hydrophilic, do not contain putative N-glycosylation sites, and are likely to be in the cytoplasmic portion of the cell. Immediately beneath the membrane-spanning region, the cytoplasmic domain is marked by a pair of arginine residues, and determination of

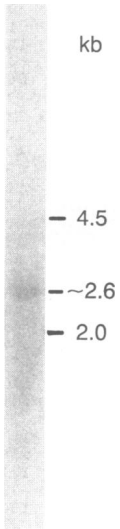

FIG. 3. Northern blot analysis. Poly(A)$^+$ RNA (10 μg) from human bone marrow was hybridized with a ^{32}P-labeled 1.9-kb fragment from clone 191.

the net charge difference of the 15 amino acids flanking the membrane-spanning region, shows a negative difference between the C- and N-terminal regions, consistent with Kell protein having its N terminus in the cell cytoplasm, and the C terminus being extracellular (17).

In agreement with carbohydrate analysis of Kell protein (5, 6), there are six possible N-glycosylation sites at amino acid positions 94, 115, 191, 345, 627, and 724.

Kell antigens are inactivated by high concentrations of dithiothreitol (18) and by treating erythrocytes with 2-aminoethylisothiouranium bromide (19). In addition, dithiothreitol mixed with papain inactivates Kell antigens (20), and mixtures of chymotrypsin and trypsin, but not the individual enzymes, destroy Kell antigenic activity (21). Taken together, these results suggest that Kell is folded, by disulfide interactions, and that its antigenic activity is destroyed by reduction and proteolytic cleavage. Consistent with this hypothesis, 16 cysteines are present in the deduced amino acid sequence. None of the cysteines appear in the cytoplasmic domain; 1 occurs in the membrane-spanning region, and the other 15 are located in the extracellular segment. A cluster of 5 cysteines occurs near the membrane-spanning site, and another cluster exists at the C-terminal portion (see Fig. 4).

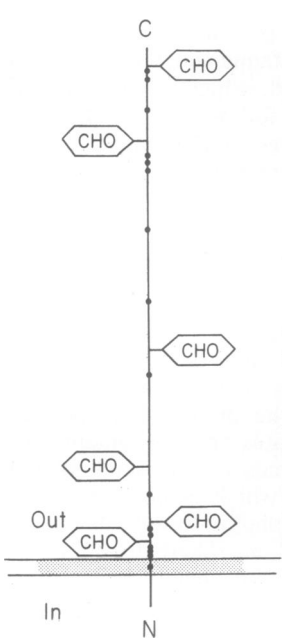

FIG. 4. Hypothetical scheme of Kell protein showing its orientation on the membrane, location of cysteine residues, and carbohydrate side chains. The cysteine residues are represented as dots, and the carbohydrate side-chains are represented as CHO. N shows the N terminus, and C indicates the C terminus of the protein. The membrane is shaded, and the cytoplasmic area (In) and extracellular space (Out) are shown.

Kell glycoprotein has several possible zinc-binding sites. These putative sites occur at a Leu-Gly-Pro-His sequence at positions 211–214 and at a neighboring histidine at position 219. Other zinc-binding sites are a pentamer sequence His-Glu-Leu-Leu-His (amino acids 581–585), which is a consensus sequence [His-Glu-(Iso-Leu-Met)-Xaa-His] with zinc-binding properties common to catalytic sites of endopeptidases from bacteria to mammals (22–24). A computer-based search also indicated that Kell protein has two overlapping leucine zipper regions, starting at amino acids 583 and 590. The heptad array of leucine residues and clusters of four to six cysteines are commonly thought involved in binding protein to DNA (25). The cysteine clusters, like zinc-finger motifs, require zinc to bind to DNA (26, 27). However, these motifs are not peculiar to gene regulatory proteins and are also found in mammalian hormone receptors. The leucine zipper domain has been postulated to be involved in protein–protein interactions (25). It should be noted that Kell antigen expression on the erythrocyte surface is related to that of another blood group, Kx. Absence of Kell antigens in Ko cells leads to enhanced Kx activity. When Kx is not expressed, as in McLeod erythrocytes, Kell antigenic activity is diminished (2). This result suggests that interactions between these proteins may affect their expression on the membrane.

Immunoblot Analysis. Further evidence, that the amino acid sequence deduced from the isolated cDNA corresponded to Kell protein, was obtained by immunizing rabbits to a synthetic 30-amino acid peptide from the C-terminal segment (amino acids 702–732). The antibody reacted, on an immunoblot, with authentic Kell protein isolated by immunoprecipitation from intact K:7 erythrocytes (see Fig. 5). The rabbit antibody also reacted with a 93-kDa protein present in total erythrocyte proteins from Kell-positive erythrocytes but did not react with proteins from membranes of Ko (null) cells. These results, together with the presence of three known Kell sequences in the deduced sequence, established the authenticity of the isolated cDNA.

Homology of Kell Protein with Neutral Endopeptidases. A computer FASTA search (28) of the entries in GenBank (release 65), EMBL (release 25), Swiss-Prot (release 16), and GenPept (release 60) showed that Kell cDNA has homology with rat and rabbit neutral endopeptidases 24.11 (EC

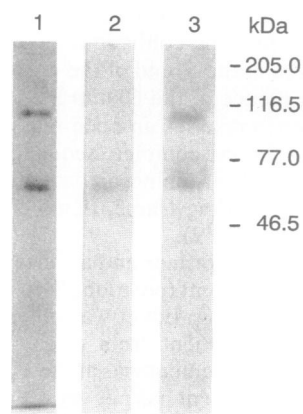

FIG. 5. Immunoblot analyses. Kell protein was isolated by immunoprecipitation from K:7 erythrocytes with a human alloimmune antibody to K:7. Lanes: 1, Coomassie-stained SDS/PAGE gel, showing an upper Kell protein band and a lower band from IgG; 2 and 3, immunoblot analyses. Preimmune serum (lane 2) and rabbit antibody to a peptide derived from the 30 C-terminal amino acids deduced from Kell cDNA (lane 3) were used. ^{125}I-labeled goat anti-rabbit IgG was used as a second antibody, and as shown in lane 2, it cross-reacts with human IgG. The peptide antibody (lane 3) reacts with the upper (Kell) band, as shown by the autoradiogram.

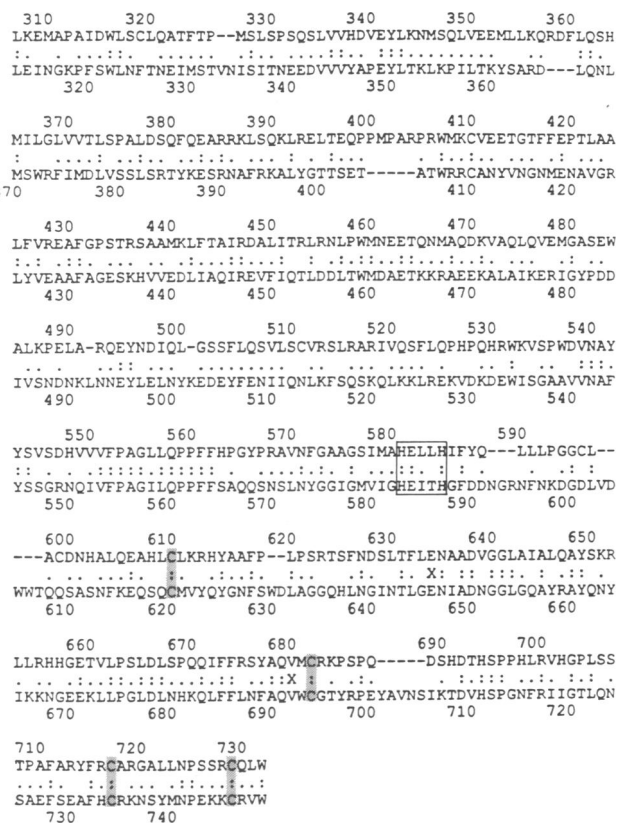

FIG. 6. Homology between CALLA and Kell proteins in a segment around the zinc-binding endopeptidase catalytic site. A 425-amino acid segment of Kell protein (upper sequence) is aligned with that of CALLA. Putative pentapeptide consensus zinc-binding and catalytic sites of endopeptidases are boxed. Alignment of four cysteine residues is shaded. Double dots (:) represent exact amino acid matches, and single dots (·) represent a match with a conservatively replaced amino acid.

3.4.24.11, enkephalinase) and with human common acute lymphoblastic leukemia antigen (CALLA). These are a family of membrane-associated proteins that span the membrane once. These endopeptidases are zinc-binding glycoproteins that are broadly distributed in many cell types and have wide substrate specificity. These endopeptidases process and inactivate a variety of peptide hormones, such as the enkephalins, oxytocin, bradykinin, angiotensins I and II, substance P, and neurotensin (29–32). Kell protein has both structural and sequence homology with this family of proteins.

Although most amino acid sequence homology of Kell and CALLA occurs in a 445-amino acid overlap in the extracellular portion of the proteins, there is also structural homology in the flanking areas near the membrane-spanning site. Both proteins have small hydrophilic cytoplasmic segments, but that of Kell is larger (47 amino acids) than that of CALLA (25 amino acids). Placing the two membrane-spanning domains in the same position aligns four Kell cysteines (residues 77, 82, 100, and 108) with four CALLA cysteines.

The homology between Kell and CALLA sequences is most striking in the segment that precedes the active catalytic site of endopeptidases. However, structural similarities also appear in regions closer to the C terminus of both proteins. A set of four cysteines in Kell and CALLA sequences can be aligned; this alignment places the putative catalytic sites of Kell (His-Glu-Leu-Leu-His) and CALLA (His-Glu-Iso-Thr-His) in juxtaposition. Preceding these pentamer amino acid sequences is 59% base identity in a 152-bp region (data not shown) and 25% amino acid identity in a 445-amino acid overlap (see Fig. 6). These structural and sequence similarities indicate that Kell protein is probably a member of the zinc neutral endopeptidase family.

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