The Kell blood group is a highly polymorphic system containing over 20 different antigens borne by the protein Kell, a 93-kDa type II glycoprotein that displays high sequence homology with members of the M13 family of zinc-dependent metalloproteases whose prototypical member is nephrilysin. Kell K1 is an antigen expressed in 9% of the Caucasian population, characterized by a point mutation (T193M) of the Kell K2 antigen, and located within a putative N-glycosylation consensus sequence. Recently, a recombinant, non-physiological, soluble form of Kell was shown to cleave Big ET-3 to produce the mature vasoconstrictive peptide. To better characterize the enzymatic activity of the Kell protein and the possible differences introduced by antigenic point mutations affecting post-translational processing, the membrane-bound forms of the Kell K1 and Kell K2 antigens were expressed either in K562 cells, an erythroid cell line, or in HEK293 cells, a non-erythroid system, and their pharmacological profiles and enzymatic specificities toward synthetic and natural peptides were evaluated. Results presented herein reveal that the two antigens possess considerable differences in their enzymatic activities, although not in their trafficking pattern. Indeed, although both antigens are expressed at the cell surface, Kell K1 protein is shown to be inactive, whereas the Kell K2 antigen binds nephrilysin inhibitory compounds such as phosphoramidon and thiophen on with high affinity, cleaves the precursors of the endothelin peptides, and inactivates members of the tachykinin family with enzymatic properties resembling those of other members of the M13 family of metalloproteases to which it belongs.

The Kell blood group is a highly polymorphic system containing over 20 different antigens, many of which are organized in five antithetical sets with opposing high and low incidence while others remain independently expressed. One of the best characterized such set of antigens is also the first to be identified and is composed of the low prevalence K11 antigen (K, Kell), expressed in 9% of the Caucasian population, linked to maternal alloimmunization and hemolytic disease of the newborn. Its antithetical form is K2 (k, cellano), and it is expressed in over 90% of the population (1, 2). The complex Kell blood group was long known as carried by a single gene (KEL) coding for a 93-kDa surface-exposed glycoprotein expressed on red cell membranes where the Kell protein has been shown to be covalently linked to a polytopic membrane protein of 37 kDa, Kx. The absence of Kx leads to a pathological condition known as McLeod syndrome, which associates to red cell acanthocytosis as well as muscular and neurological disorders (3, 4). The molecular cloning of the gene coding the Kell protein (5) has opened new paths in the study of the Kell blood group system as the molecular basis of the various antigenic forms were brought to light and with the revelation of its high degree of sequence homology with the members of the M13 family of zinc-dependent metalloproteases.

This family of proteases, whose prototypical member is nephrilysin (NEP, EC 3.4.24.11), is involved in the activation or inactivation of various messenger peptides by their cleavage on the amino-terminal side of hydrophobic residues (6). Besides NEP, the M13 family also comprises the endothelin-converting enzymes ECE-1 and ECE-2 (7, 8), the phosphate-regulating neutral endopeptidase on the X chromosome (9), endothelin-converting enzyme-like 1 (also known as damage-induced neutral endopeptidase) (10–12), NEP2 (also termed secreted endothelin-converting enzyme-like 1 or NEP2) (13–15), and Kell (1, 2, 5). These metalloproteases are type II integral membrane glycoproteins sharing a high degree of sequence homology, particularly within their large COOH-terminal domain, which contains their active site along with the consensus zinc-binding motif HEXXH. The recent disclosure of the tri-dimensional structure of NEP as revealed by x-ray crystallography (16) has enabled modeling studies of other members of the family that suggest that their overall three-dimensional structures are also highly conserved inside as well as outside the active site (16, 17, 19).

The tertiary structure of these metalloproteases is maintained by at least 10 conserved cysteine residues forming disulfide bridges. Although the NEP structure has been shown to

neprilysin; ECE, endothelin-converting enzyme; PBS, phosphate-buffered saline; PBS/BSA, PBS containing 0.5% bovine serum albumin (w/v); AMG, amidomethylcoumarin; HEK, human embryonic kidney; NglyF, N-glycosidase F; Endo H, endoglycosidase H; ER, endoplasmic reticulum; TGN, trans-Golgi network; sol, solubilized fraction; Glut, glutaryl; ACE, angiotensin-converting enzyme; RBC, red blood cell; MEGS, 4-morpholineethanesulfonic acid; Big-ET, big-endothelin; Suc, succinyl.
be held by six such bridges, it seems that five may be sufficient to confer a NEP-like structure and enzymatic profile because five may at best exist in NEP2, the closest NEP homologue (17, 20). Whereas the cysteine residues of NEP are all involved in intramolecular disulfide bridges (21), other members of the family have been shown to form intermolecular complexes through unpaired cysteine residues. For example, ECE-1 forms a homodimer through its non-conserved Cys112 residue (22) with subtle changes to the kinetic properties of the dimerized protease compared with the monomer (23). Finally, Kell can form a heterodimer by linking Kx to its Cys72 residue (24). Although the function of Kx remains unknown, the formation of the Kell-Kx complex seems to occur in the ER in COS cells (25) and the lack of Kx, which leads to the McLeod phenotype, is also associated with decreased Kell antigenic reactivity on red blood cells (26).

Other conserved features include post-translational modifications and, in particular, glycosylation, which has been shown to be relevant to the trafficking and enzymatic activity of these proteases (27, 28). The predicted sequence of the Kell protein possesses five potential N-glycosylation sites. Among the identified point mutations of the Kell system so far, one may affect such a process, that of Kell K1, which is defined by a C to T base substitution in exon 6 leading to the replacement of a threonine by a methionine residue (T193M) within a putative N-glycosylation consensus sequence Asn-X-Thr/Ser. This mutation could alter the polysaccharide content of Kell and lead to a change in protein conformation and/or trafficking into the cellular machinery (29), which could in turn affect its function.

NEP and NEP2 are rather promiscuous enzymes in vitro efficiently cleaving many small messenger peptides into inactive fragments (28). Although it has also been shown to cleave other messenger peptides in vitro (30), ECE-1 seems more specific insofar as it remains the only enzyme involved in the maturation of endothelin-1 by cleaving the inactive Big-endothelin-1 (Big-ET) intermediate at its Trp31-Val72 bond. Endothelins are potent vasoconstrictor peptides produced from three distinct genes, which code for distinct precursors and intermediates termed Big-ET-1, Big-ET-2, and Big-ET-3, the most abundant of which seems to be Big-ET-1 (31), the preferred substrate of ECE-1 (7).

In vivo, the use of specific inhibitors of nephrilysin, e.g. thiorphan, has suggested NEP to be involved in the inactivation of enkephalins (32), tachykinins (33), and atrial natriuretic peptide (34). As the enzymatic specificity and pharmacological profile of novel enzymes of the family are being characterized, however, the true physiological functions of each individual enzyme and of NEP, in particular, can be put into question (20, 28, 35). Indeed, as members of a given family of proteins may have overlapping functions, it now becomes essential to study and characterize the orphan enzymes of this family.

Whereas Kell as a blood group is well characterized in terms of antigenicity, its physiological function remains unclear. Even though Kell is mainly expressed in erythroid tissues like red blood cells, bone marrow, and fetal liver, Northern blot analysis has recently shown that Kell is also present in non-erythroid cells, primarily in testis and heart (36), and dot blots further suggest its possible expression in brain (36–37). Furthermore, a recombinant soluble form of Kell (sKell) without its intracellular and membrane-spanning domains was expressed using insect cells and shown to possess an endothelin-convert- ing enzyme activity, cleaving all three intermediates with a preference toward Big-ET-3, which was cleaved at its Trp21-Ile72 bond to produce mature ET-3 (38).

In order to further characterize the enzymatic activity and specificity of the Kell protein and particularly its physiological forms in physiologically relevant systems, two antithetical and common antigenic forms of the blood group, K1 and K2, were expressed in either K562 cells, an erythroleukemia cell line, or in the endothelial HEK293 cells. The study of their subcellular distributions therein shows them to be identical despite their confirmed difference in glycosylation. However, whereas both K1 and K2 were expressed at the cell surface in both cell lines, only the Kell K2 protein possesses a metalloprotease activity that is characterized in detail using a model substrate as well as various bioactive messenger peptides. These results show the Kell protein bearing the K2 antigen to be a metalloprotease-inhibited by metal chelators and by the generic M13 family inhibitor phosphoramidon. The Kell K2 protein bound transition-state inhibitors known to be NEP-specific, such as thiorphan (39, 40), with very high affinity. Finally, the previously suggested Big-ET-3 maturing activity of Kell (38) is confirmed for the Kell K2 antigen and novel physiological substrates identified.

EXPERIMENTAL PROCEDURES

Materials—Synthetic fluorescent and bioactive peptides were purchased from Bachem (Weil am Rhein, Germany). Phosphoramidon, brefeldin A, monensin, and saponin were from Sigma-Aldrich (Saint Quentin Fallavier, France), whereas thiorphan, captopril, omapatrilat, and compound I were generous gifts from Bioproteq (Paris, France). All of the products used for cellular culture were from Invitrogen (Cergy Pontoise, France) unless otherwise stated.

Kell K1 and Kell K2 Constructs—Starting material for PCR amplification of the Kell coding sequence was a human bone marrow cDNA library (Clontech Palo Alto, CA). Forward and reverse primers used for amplification were 5'-TGGGGGATCCGGCACATGAACTGGGGA-CAAAGG-3' and 5'-ACCCCTTCTAGAGTGGCATTCTTTGTAACCAAGA-G3' respectively (the cloning sites are underlined and italicized). The purified PCR product was digested with BamHI and XbaI, and overhanging 4 bases were filled with T4 DNA polymerase for blunt EcoRV cloning in pcDNA3 expression vector (Invitrogen). All of the enzymes were from New England Biolabs (Beverly, MA). Sequence verification demonstrated that the amplified Kell sequence corresponded to the common R2 antigen.

To obtain the K1 sequence, site-directed mutagenesis was performed using the Stratagene QuikChange kit and an adequate pair of primers (the sequence of the sense primer was 5'-CTTCTAACCCTGCTT-GAGACTTCTG-3', the mismatch is indicated with underlined italics).

Cell Culture and Transfections—HEK293 cells were cultured in Ham's F-12 medium, and K562 cells were cultured in RPMI 1640 medium, both supplemented with 10% (v/v) fetal bovine serum. The pcDNA3.1 vector (control) and Kell K1 and Kell K2 constructs were transfected into HEK293 cells using Superfect (Qiagen, Courtaboeuf, France) as previously described (28), whereas K562 cells were transfected by electroporation. K562 and HEK293 clones expressing Kell K1, Kell K2, or the empty vector were selected after 2 weeks in culture medium supplemented with 800 µg/ml Genetecin and maintained in culture medium containing 200 µg/ml antibiotic. Routinely, HEK293 cells were scraped 4–5 days after seeding and harvested in ice-cold HEPES buffer (50 mM, pH 7), whereas K562 cells were collected by centrifugation. After cell disruption with a Polytron (Kinematica, Lucerne, Switzerland), membranes were obtained by centrifugation (twice at 50,000 × g for 40 min each), resuspended in HEPES buffer (50 mM, pH 7, 0.15 mM NaCl, 0.01% Triton X-100) at a final concentration of 1 µg/µl, and solubilized in HEPES buffer containing 2% Brij-58: 1% Triton X-100 for 16 h at 4 °C, a combination of detergents that has previously been shown not to affect the enzymatic activity of metalloproteases of the M13 family (28). The non-soluble fraction was subsequently removed by centrifugation.

Endoglycosidase Digestions—HEK293 or K562 membranes (10 µg) expressing either Kell K1 or Kell K2 antigens were heat-denatured in SDS and incubated with either recombinant N-glycosidase F (NglyF, 2 units) or endoglycosidase H (EndoH, 2.5 milliunits) for 16 h at 37 °C according to the supplier's instructions (Roche Applied Science, Meylan, France). Controls were incubated without enzyme, and samples were then processed for immunoblotting.

Red blood cells (RBCs) with known phenotypes were obtained from the Centre National de Reference pour Les Groupes Sanguins (Paris, France). They were washed in digestion buffer (112.5 mM NaCl, 25 mM PO4, 50 mM EDTA, 5 mM glucose, 2 mM 2-aminomethyl-benzosulfonyl-
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fluorohydrochloride, 2 mM Pefabloc, pH 7.5), diluted 10 times in the same buffer, and incubated overnight at 37 °C with 10 units of \( \text{N}-\text{glycosidase F} \) in a final volume of 230 μl. Total digestion of the samples was verified by running an aliquot of the denaturing acrylamide gel followed by immunoblotting, whereas the remaining cells were sorted using various antigen-specific antibodies (see below).

**Immunofluorescence**—Cells were seeded on 14-mm collagen-coated coverslips and fixed after 24 h with 4% paraformaldehyde for 12 min. Fixed cells were washed with PBS, and nonspecific binding was saturated with preincubation buffer (10% decomplemented fetal calf serum, 0.2% BSA, 0.05% saponin in PBS). The Kell-specific monoclonal antibody 5A11 directed toward the common, intracellular, amino-terminal region of the protein (41) was diluted (1/1000) in preincubation buffer and incubated for 16 h at 4 °C. Cells were subsequently washed with PBS and incubated for 1 h at room temperature with the appropriate secondary antibody (goat antibody directed against rabbit IgG coupled to Cy3) and coverslips were mounted with Mowiol and observed with a Leica confocal microscope.

**Fluorescence-activated Cell Sorting**—HEK cells (5 × 10⁴) were resuspended in 100 μl of PBS/BSA (phosphate-buffered saline containing 0.5% bovine serum albumin (w/v)). 100 μl of antibodies in PBS/BSA were added at saturating concentrations to the cell suspension, and the mixture was incubated for 90 min at 25 °C. After washing in PBS/BSA, the cells were resuspended with 100 μl of 1/1000 dilution of fragments of goat anti-mouse or anti-human IgG (Beckman Coulter, Villepinte, France) for 45 min at 25 °C. After washing with PBS, cells were suspended in 500 μl of PBS/BSA containing 0.2 mg/ml propidium iodide. Analysis was performed on a FACSCalibur flow cytometer (BD Biosciences) using the CellQuest software. Propidium iodide-positive dead cells were excluded from analysis. HEK cells expressing the Kell K2 antigen were sorted with the mouse monoclonal F7 clone (obtained from Dr. P. Rubinstein, the New York Blood Center), whereas K1 antigen-expressing HEK cells were identified according to their reactivity toward the human T27S antibody (42).

**Cell Treatments**—HEK293 cell lines expressing either Kell K1 or Kell K2 antigens were brought to 80–90% confluency in 150-mm plates. Treatment with 16 h of 10 μM protease inhibitor with 5 μM of transport blocker brefeldin A or 5 μM of 50 μM Glut-FAAF-AMC or 0.75, 0.5, 0.25, and 0 M (NH₄)₂SO₄ supplemented with 10% NaCl and 0.05% Triton X-100. Fractions were immunoblotted with the Kell-specific antibody, and reactive fractions were pooled and concentrated on a Centricon YM 100 device (Millipore, Brumath, France), whereas the (NH₄)₂SO₄ buffer was replaced by 50 mM HEPES, pH 7, buffer containing 0.15 M NaCl and 0.01% Triton X-100. Protein from the lectin fraction of either Kell K1 or K2 lectin purification step (concanavalin A) and diluted in the reaction buffer (50 mM HEPES, pH 7, containing 0.15 M NaCl, 0.01% Triton X-100).

For \( K_a \) determinations, the incubation mixtures were comprised of 10 μg of total protein from the lectin fraction of either Kell K1 or K2 HEK293 cell membranes and from 1 to 128 μM Glut-FAAF-AMC or Suc-AAP-AMC diluted in 100 μl of reaction buffer. Incubations proceeded for 37 °C for 60 min followed by a 60-min incubation at 56 °C with an excess of aminopeptidase M to release the fluorescent AMC moiety. Inhibitory potencies were determined as follows, 20 μM Glut-FAAF-AMC and 10 μg of total protein of either Kell K1 or K2 lectin fraction were diluted in 100 μl of reaction buffer and incubated with inhibitory compounds at concentrations ranging from 0.01 nM to 10 μM. Incubations were performed as described above, i.e. 37 °C for 60 min followed by a 60-min incubation with aminopeptidase M. Inhibitory potencies (\( K_i \)) of the various compounds were calculated from the IC₅₀ using the Cheng and Prussoff equation (44).

**HPLC and Electrospray Mass Spectrometry**—The substrate specificities of Kell K1 and Kell K2 against bioactive peptides were explored using the three-step semi-purified preparations of either enzyme. Bioactive peptides (10 μM, listed in Table II) were incubated with 5 μg of Kell K1 or Kell K2 expressing HEK293 cells in PBS, pH 7, containing 0.025% Triton X-100 at 37 °C for 2 h. The reactions were terminated by adding 5 μl of acetic acid 1N. The specific activity of each reaction against the substrate was verified by preincubating 100 nm inhibitory compound 1 (10 min at 37 °C) with the enzyme before the addition of each peptide. For \( K_e \) determinations, 5 μg of protein from the Centricon YM 100 fraction of either Kell K1 or K2 were incubated with increasing concentrations (1.25–160 μM) of substrate, i.e. substance P and neurokinin A diluted in 50 μl of 50 mM HEPES, pH 7, supplemented with 0.15 M NaCl.

All of the samples were analyzed by mass spectrometry coupled to HPLC. Electrospray ionization mass spectra were acquired on a Finnigan LCQ Advantage ion trap mass spectrometer. On-line HPLC separation was achieved with a 5 μm, 150 mm, Waters) packed with dC18 absorbent (5 μM, 300 Å) (mobile phase A, 0.1% formic acid; mobile phase B, 80% acetonitrile, 0.1% formic acid). The first 4 min at 0% B were derivatized to waste. The following non-linear gradient was then used: 20–30% mobile phase B over 10 min followed by 30–70% B over 10 min (flow rate: 250 μl/min). The column was washed within 5 min with 100% solvent B before resequilibration with mobile phase A. Substrates and metabolite ions were sequentially fragmented into the ion trap by collision-induced dissociation using an isolation width of 3 and a relative collision energy of 35%. Mass spectrometry/mass spectrometry fragmentation data allowed an unambiguous identification as well as quantification of the different substrates and their metabolites using external standards and the LCQuan/Excalibur software.

**RESULTS**

Expression of Kell K1 and Kell K2 Antigens in Mammalian Hematopoietic and Endothelial Cells—Kell K1 and Kell K2 antigens were expressed in two types of human cell lines: K562 cells, which are hematopoietic malignant cells expressing endogenous Kell, and HEK293 cells, kidney fetal cells devoid of Kell expression (as observed by reverse transcription-PCR, data not shown). The transfection of Kell K1 and Kell K2 constructs in K562 and HEK293 cells resulted in the expression of a protein of ~115 kDa for the K2 antigen and ~110 kDa for K1 as revealed by immunoblotting experiments using the...
immunoblotted with the Kell-specific antibody (5A11) recognizing both isoforms of Kell. 

Kell K1-transfected HEK293 cells and Kell K2-transfected HEK293 cells were immunoblotted with the Kell-specific antibody (5A11) recognizing transfected HEK293 cells and Kell K2-transfected HEK293 cells were K2 antigens.

K562 and HEK293-transfected cells with either Kell K1 or Kell cells (K1-transfected K562 cells (left panel) and Kell K2-transfected K562 cells (right panel) were digested with either NglyF or Endo H and immunoblotted with the Kell-specific antibody. C, crude membrane preparations (10 μg) of Kell K1-transfected HEK293 cells (left panel) and Kell K2-transfected HEK293 cells (right panel) were digested with either NglyF or Endo H and immunoblotted with the anti-Kell protein-specific antibody.

The results, particularly in HEK cells, confirm the glycosylation of Asn191-X-Thr193 consensus sequence; therefore, its substitution to a methionine results in its disappearance. The results, particularly in HEK cells, confirm the glycosylation of Asn191 of the Kell K2 protein as well as the loss of this polyasaccharide moiety in the K1 antigen of Kell reflected by its molecular weight decreased by approximately the estimated weight of a polysaccharide moiety. This result is further confirmed by the observed molecular weight shift after the removal of all the sugars by NglyF treatment, which brings both K1 and K2 antigens to an identical molecular weight, i.e. ~100 kDa (Fig. 1, B and C). Whereas N-glycosidase F removes all the sugars from the protein backbone, Endo-H cleaves oligosaccharides of the high mannose type found exclusively on proteins of the ER. Treatment of membranes from either cell line expressing either Kell K1 or Kell K2 with endoglycosidase H shows that both antigenic forms of Kell have acquired resistance to this enzyme, suggesting that they have at least reached the Golgi apparatus.

Immunofluorescence experiments using the common Kell-specific antibody, which recognizes the intracellular domain of all the antigens of the Kell protein, were performed on HEK293 Kell K1 or Kell K2 cell lines. The data in Fig. 2 show that this antibody mainly labeled HEK293 plasma membranes along with punctate intracellular structures, reflecting the labeling of the protein en route through the various compartments of the secretory pathway (Fig. 2, A and B). Although no striking difference in immunofluorescence was observed between HEK293 cells expressing either Kell K1 or Kell K2, suggesting that both antigenic forms of Kell are equally able to reach the cell surface, there may be slightly more intracellular Kell K2 immunofluorescence than Kell K1. No immunofluorescent signal was detected in HEK293 cells transfected with the pcDNA3.1 plasmid alone (data not shown). Data obtained with flow cytometry using antigen-specific extracellular antibodies further confirm the surface expression of both Kell K1 and Kell K2 in HEK293 as well as in K562 cells (data not shown).

The ensemble of these results brings to light the difference in post-translational processing between the Kell K1 and Kell K2 protein backbone but shows that this difference in glycosylation does not dramatically affect their transport in either K562 or HEK293 cells. These antigens of the Kell blood group are specifically recognized by different antibodies (2). Although this argues for a different conformation of the Kell antigens, in the case of antibodies discerning K1 from K2, one may wonder whether the K2 epitope is constituted or not by the specific K2 sugar moiety on Asn191, the removal (or absence) of which would preclude anti-K2-specific antibody binding on one hand and reveal the K1 antigenic site on the other hand.

To test this hypothesis and further investigate the possible conformational modifications induced by the T193M mutation, RBCs homozygous for either the Kell K1 or Kell K2 antigen were deglycosylated and their recognition by various antigen-specific (three for K1 and two for K2) antibodies were monitored by fluorescence-activated cell sorting. The results of these experiments (Fig. 3) reveal that the deglycosylation of either Kell antigen does not alter its binding to either antibody, suggesting that its conformation is not modified by the deglycosylation process, which moreover does not reveal any antigenic site. These observations demonstrate that the anti-K2 antibodies do not recognize the oligosaccharide on Asn191 and suggest that the anti-K1 antibodies bind an epitope distinct from the Met191 residue of the Kell K1 antigen.

Partial Purification of Kell Antigens and Model Substrate

Enzymatic Activity of Kell K1 and Kell K2 Antigens

Enzymatic activity using the following small synthetic fluorogenic peptides: MeOSuc-GLF-AMC; MeOSuc-GWM-AMC; MeOSuc-VRR-AMC; MeOSuc-NYM-AMC; MeOSuc-GP-AMC; MeOSuc-AAPM-AMC; Suc-AAF-AMC; and Glut-FAAF-AMC. Having observed a small difference in the quantity of released AMC between control and Kell K2 cells using Glut-FAAF-AMC, the
membranes were solubilized with the intent to semi-purify the preparations on a lectin column. However, Kell antigens were exclusively solubilized in HEK293 cells, precluding any further manipulation of the Kell-expressing K562 clones.

Membranes from HEK293 cells expressing vector alone, Kell K1, or Kell K2 were thus solubilized, semi-purified on concanavalin A, and again tested for their enzymatic activity against the above-mentioned list of synthetic peptides. The last two fluorogenic peptides, i.e. Suc-AAF-AMC and Glut-FAAF-AMC, were found to be hydrolyzed by Kell K2-containing material (Fig. 4B, bottom graph), whereas Kell K1-containing material (Fig. 4A, bottom graph) or HEK293-expressing pcDNA 3.1 vector (data not shown) possessed no enzymatic activity toward the different synthetic fluorogenic substrates. The cleavage of these two model substrates by Kell K2 reflects an endopeptidase activity of the K2 antigen-bearing protein, because omission of aminopeptidase M from the assays abolished the release of the fluorescent AMC molecule. This endopeptidase activity was further confirmed by HPLC and electrospray mass spectrometry analysis with the formation of Phe-AMC by Kell K2 but not by Kell K1-expressing membranes (Table II). Kinetic parameters measured for Kell K2 reveal an apparent $K_m$ of $\approx 25 \mu M$ and a $V_{\text{max}}$ of 990 pmol of AMC $\mu g$ protein$^{-1}$ h$^{-1}$ for Glut-FAAF-AMC hydrolysis (Fig. 5A). These values are comparable to those obtained for Suc-AAF-AMC hydrolysis, i.e. a $K_m$ of $\approx 65 \mu M$ and $V_{\text{max}}$ of 1032 pmol of AMC $\mu g$ protein$^{-1}$ h$^{-1}$. Hydrolysis by Kell K2 occurred with a pH optimum between 7 and 8 (Fig. 5B), whereas no proteolytic activity of the K1-bearing antigen was induced by these pH modifications (data not shown). The enzymatic activity of K2 was completely abolished by the metal chelator EDTA (1 mM), the generic phosphoramidon molecule, and by NEP-specific transition-state inhibitors, i.e. compound I (17) and thiorphan as well as by omapatrilat, a mixed NEP/ACE inhibitor. Inhibitory potencies of these compounds were in the nanomolar range with the exception of captopril, an ACE-specific compound, which displayed no detectable inhibition of Kell K2 activity (Fig. 5C and Table I). Testing the sensitivity of the Kell K2 metalloprotease...
The fluorogenic substrate Glut-FAAF-AMC (20 μM) was used to evaluate the inhibitory potencies of phosphoramidon, a generic inhibitor of the M13 family of metalloproteases, of thiorphan and compound I, NEP-specific inhibitors, of omapatrilat, a mixed NEP/ACE inhibitor as well as of captopril, an ACE-specific inhibitor (K<sub>i</sub> values were determined from data shown in Fig. 5C). Enzyme activity was completely abolished by 1 mM EDTA. Results presented in the table are the mean values of at least two independent determinations. Inhibitory potencies of these compounds against NEP were previously reported using the same protocol with Suc-AAP-AMC as a model substrate (17, 28).

| Inhibitor          | K<sub>i</sub> (nM) |
|--------------------|-------------------|
| Phosphoramidon     | 18.1              |
| Thiorphan          | 4                 |
| Compound I         | 0.7               |
| Omapatrilat        | 14.3              |
| Captopril          | >10 μM            |

**FIG. 4. Purification of Kell K1 and Kell K2 from HEK293-transfected cells.** Equal amounts of total protein from each purification step of Kell K1 (A) and Kell K2 (B) were separated on 4–12% SDS-polyacrylamide gels under reducing conditions and immunblotted with the anti-Kell protein-specific antibody (top panel). The specific activities of Kell K1 and Kell K2 were tested using the fluorogenic substrate Glut-FAAF-AMC (20 μM, bottom panel). The specific activity represents total fluorescence from which is subtracted “nonspecific” activity represented by the fluorescence observed in the presence of 1 mM EDTA or 100 nM compound I; sol., solubilized fraction; con. A, concanavalin A; phenyl seph., phenyl-Sepharose.

**FIG. 5.** Enzymatic activity of semi-purified Kell K2 using a fluorogenic model substrate. All of the enzymatic activity experiments were performed using 20 μM Glut-FAAF-AMC diluted in 50 mM HEPES buffer supplemented with 0.15 M NaCl and 0.01% Triton X-100 unless otherwise stated. For these experiments, the lectin-purified K2 preparation was used. Data are mean values from at least two independent determinations. A, the affinity constant (K<sub>i</sub>) was determined by adding increasing concentrations of Glut-FAAF-AMC (1–128 μM) to 10 μg of Kell K2 semi-purified preparation. B, pH optimum of semi-purified Kell K2 was determined using 20 μM Glut-FAAF-AMC in MES (○), HEPES (△), or Tris-HCl (■) buffer at the indicated pH values. C, Glut-FAAF-AMC (20 μM) was incubated in the presence of Kell K2 and increasing concentrations of compound I (●), thiorphan (□), omapatrilat (▲), or phosphoramidon (asterisk). The obtained K<sub>i</sub> values from the conversion of IC<sub>50</sub> values are reported in Table I. D, the effect of Zn<sup>2+</sup> on semi-purified Kell K2 activity was determined using 20 μM Glut-FAAF-AMC and increasing concentrations of ZnCl<sub>2</sub>.

against its associated metal ion Zn<sup>2+</sup> shows it to respond in a biphasic mode, in that low concentrations (up to 10 μM) promote, whereas high concentrations of ZnCl<sub>2</sub> inhibit, Kell K2 protein activity (Fig. 5D).

**Trafficing and Activation of Kell K2**—To explore whether Kell K2 is immediately translated into an active enzyme or whether it is activated en route through the secretory pathway, the effect of blocking Kell K2 in various subcellular compartments was investigated. To this end, cells expressing the active Kell K2 antigen were subjected to various treatments known to affect transport through the secretory pathway (Fig. 6). Temperature blockade causes retention in specific compartments and thus affects post-translational processes. In particular, 15 °C blocks are known to prevent exit of secretory proteins from the ER, whereas incubation of cells at 20 °C blocks exit from the trans-Golgi apparatus (TGN) (45). Such temperature blockade of Kell K2-expressing HEK293 cells at either 15 or 20 °C for 8 h resulted in a significant 45% loss in total recovered enzymatic activity, a loss that probably corresponds to Kell K2 protein retained in either the ER or TGN, which appear as compartments in which Kell K2 is inactive. Chemical treatments with brefeldin A or monensin compounds, which block the exit of secretory proteins from the TGN, albeit with different molecular mechanisms (45–47), further confirm these results. Indeed, treatment with either drug for a period of 16 h resulted in an almost 70–95% loss of activity with brefeldin A or monensin, respectively (Fig. 6), results that remain in line with the ones obtained by temperature blockade because they represent approximately twice the loss of activity over exactly twice the treatment time.

**Hydrolysis of Natural Peptides**—For the study of the substrate specificities of both Kell K1 and Kell K2, the lectin fraction of HEK293 membranes expressing either the vector alone or the K1 or K2 antigen was subjected to two additional steps of purification to avoid contamination by unrelated peptidases (Fig. 4). The purification of the antigens was followed
both by immunoblotting (Fig. 4, top panel) and model substrate activity (bottom panel). For the Kell K2 preparation, this three-step procedure resulted in a 125-fold purification of the protein of interest, as measured by its specific enzymatic activity toward Glut-FAAF-AMC using 100 nM inhibitory compound I to quantify nonspecific proteolytic activity. This preparation was used to test the substrate specificities of the studied Kell antigens toward various natural bioactive peptides by HPLC and mass spectrometry. To this end, each peptide tested was incubated with equal amounts of semi-purified membranes of control, Kell K1, or Kell K2 HEK293 cells. Control reactions for cleavage specificity included parallel incubations of peptide alone as well as with 100 nM inhibitory compound I.

The results obtained show that the membrane-bound form of Kell K2 is capable of cleaving Big ET-1 and Big ET-3 at their Trp<sup>21</sup>-Val<sup>22</sup> and Trp<sup>21</sup>-Ile<sup>22</sup> amide bonds, respectively, a cleavage that is completely blocked in the presence of compound I (100 nM) (data not shown). However, no mature ET-1 or ET-3 was formed in the presence of Kell K1 or of membranes purified from HEK cells transfected with the vector alone, confirming the enzymatic inactivity of the Kell K1 antigen on natural bioactive peptides. Kell K2 was also found to specifically cleave yet unidentified substrates, i.e. substance P (Fig. 7) and neurokinin A (Fig. 8), both belonging to the tachykinin family of messenger peptides. The major cleavage site for substance P, representing 69% substrate consumption, was found to be located between residues Gly<sup>9</sup> and Leu<sup>10</sup> of this undecapeptide. Two other amide bonds of this messenger peptide are also attacked, i.e. Gln<sup>6</sup>-Phe<sup>7</sup> and Phe<sup>7</sup>-Phe<sup>8</sup>, but to a lesser extent. Neurokinin A was cleaved at a single site, Val<sup>2</sup>-Leu<sup>10</sup>, releasing (as in substance P) the amidated Leu-Met-NH<sub>2</sub> dipeptide. The specificity of these cleavages was confirmed by their complete inhibition by compound I. Although the K1 antigen was also tested in parallel with Kell K2, no proteolysis of these messenger peptides was ever observed with this preparation, suggesting once again that the K1 antigen of Kell is inactive. Parallel experiments using material obtained from HEK293 cells expressing the pcDNA3.1 vector contained no specific enzymatic activity.

To compare the affinities of the various peptides tested toward Kell K2, <i>K<sub>m</sub></i> and <i>V<sub>max</sub></i> values were obtained using equal quantities of enzyme preparation determined as picomoles of Glut-FAAF-AMC synthetic peptide, membranes from either antigen expressing membranes, the membrane fractions expressing either antigen. Small synthetic fluorogenic peptides were screened to identify a model substrate. Interestingly, whereas membranes of K562 and HEK293 cells expressing the Kell K2 antigen were found to possess specific enzymatic activity compared with membranes from cells expressing the vector alone using the Glut-FAAF-AMC synthetic peptide, membranes from either cell line expressing the K1 antigen remained devoid of activity. To confirm this difference between the two antigens and to better characterize the activity contained in the K2-expressing membranes, the membrane fractions expressing either antigen were subjected to a purification protocol, which was followed by immunoblotting using a common Kell-specific antibody and by enzymatic activity using the above mentioned fluorogenic peptide (Fig. 4). As for the purification of any membrane-associated protein, the first step relies on proper solubilization, which in the case of enzymes must not affect enzymatic activity, and as demonstrated here, polyc saccharide content, K1 still attains a conformation validated within the ER compartment, a conformation that, at the cell surface, possesses superior antigenicity than that of Kell K2 (29). Thus it appears that there may be many “correct” conformations to one protein or that subtle conformational changes cannot be distinguished within the ER, even though both antigens may display different retention times within this compartment (Fig. 2). Nevertheless, the ensemble of the results point to the fact that the T193M mutation not only renders impossible the transfer of a glycosidic chain to Asn<sup>191</sup> but induces a conformational change characterizing the K1 antigen of Kell. Indeed, this hypothesis is not only comforted by the existence of specific K1 and K2 antibodies but also by the demonstration that recognition of specific Kell K1 and K2 antigens by these latter antibodies is independent of glycosylation, at least once the process is completed i.e. once the protein is folded and has reached its appropriate subcellular compartment (Fig. 3).

To characterize the enzymatic activity as well as to draw an accurate pharmacological profile of Kell and of its two studied antigens, small synthetic fluorogenic peptides were screened to identify a model substrate. Interestingly, whereas membranes of K562 and HEK293 cells expressing the Kell K2 antigen were found to possess specific enzymatic activity compared with membranes from cells expressing the vector alone using the Glut-FAAF-AMC synthetic peptide, membranes from either cell line expressing the K1 antigen remained devoid of activity. To confirm this difference between the two antigens and to better characterize the activity contained in the K2-expressing membranes, the membrane fractions expressing either antigen were subjected to a purification protocol, which was followed by immunoblotting using a common Kell-specific antibody and by enzymatic activity using the above mentioned fluorogenic peptide (Fig. 4). As for the purification of any membrane-associated protein, the first step relies on proper solubilization, which in the case of enzymes must not affect enzymatic activity, and therefore be “gentle”. Kell had previously been shown to remain in the Triton X-100 insoluble fraction both in K562 expressing endogenous or recombinant Kell and in erythroid progenitor cells (51). Therefore, it was not surprising to observe both K1

\[ \text{Km} = \frac{1}{V_{max}} \]

\[ \text{V}_{\text{max}} = \frac{1}{Km} \]

\[ \text{K}_{\text{m}} = \frac{1}{\text{V}_{\text{max}}} \]

2 A. Clapéron and T. Ouimet, unpublished results.

Expression of the Kell K1 and Kell K2 antigens in these cell lines reveals that both antigens are glycosylated proteins of 110 and 115 kDa, respectively. Because the K1 antigen of Kell is characterized by a point mutation within an N-glycosylation consensus site, Asn-X-Thr/Ser<sup>193</sup>, the results presented herein suggest that the asparagine residue in position 191 of K1 is not glycosylated, unlike that of K2. Glycosylation within the ER is a conserved process involved in the acquisition of the correct tertiary structure of glycoproteins. Misfolded glycoproteins usually aggregate in the ER and are eventually targeted to the proteasome degradation pathway (48). However, there are exceptions to this rule, as misfolded proteins can be transported to their correct subcellular location (48, 49). Indeed, the altered post-translational processing of the K1 antigen does not as demonstrated here cause its retention within the ER, because K1 acquires endo H resistance and exits the TGN (Fig. 1, B and C) to reach the cell surface as does K2 (Fig. 2). This result is in accordance with previously published studies that have shown that K1 antigenic sites were found in equivalent quantities to K2 sites on heterozygous red cells (50), suggesting that, despite its modified sequence and as demonstrated here, polyc saccharide content, K1 still attains a conformation validated within the ER compartment, a conformation that, at the cell surface, possesses superior antigenicity than that of Kell K2 (29).

To characterize the enzymatic activity as well as to draw an accurate pharmacological profile of Kell and of its two studied antigens, small synthetic fluorogenic peptides were screened to identify a model substrate. Interestingly, whereas membranes of K562 and HEK293 cells expressing the Kell K2 antigen were found to possess specific enzymatic activity compared with membranes from cells expressing the vector alone using the Glut-FAAF-AMC synthetic peptide, membranes from either cell line expressing the K1 antigen remained devoid of activity. To confirm this difference between the two antigens and to better characterize the activity contained in the K2-expressing membranes, the membrane fractions expressing either antigen were subjected to a purification protocol, which was followed by immunoblotting using a common Kell-specific antibody and by enzymatic activity using the above mentioned fluorogenic peptide (Fig. 4). As for the purification of any membrane-associated protein, the first step relies on proper solubilization, which in the case of enzymes must not affect enzymatic activity, and therefore be “gentle”. Kell had previously been shown to remain in the Triton X-100 insoluble fraction both in K562 expressing endogenous or recombinant Kell and in erythroid progenitor cells (51). Therefore, it was not surprising to observe both K1

2 A. Clapéron and T. Ouimet, unpublished results.
| Substance          | Sequence   | RT (min) | Theoretical mass |
|--------------------|------------|----------|------------------|
| Substance P        | RPKPQFFGLM-amide | 17.4     | 1347             |
| Substance P 1-7    | RPKPQQ      | 9.2      | 900              |
| Substance P 10-11  | LM-amide    | 9.2      | 261.9            |
| Substance P 7-11   | FFGLM-amide | 21.5     | 613              |

FIG. 7. Hydrolysis of substance P by Kell K2 but not Kell K1. After a three-step purification, Kell K1 and Kell K2 were incubated using 10 μM substance P in HEPES buffer for 2 h at 37 °C. 10 μl of the reaction mixture were subjected to HPLC-electrospray mass spectrometry as described under “Experimental Procedures.” Control panels (top) represent the peptide incubated alone for 2 h at 37 °C. Numbers within parentheses represent the number of charges held by the peptide.
Hydrolysis of neurokinin A by Kell K2 but not Kell K1. The hydrolysis of neurokinin A by either Kell K1 or Kell K2 was also studied. Control panels (top) represent the peptide incubated alone for 2 h at 37°C. Numbers within parentheses represent the number of charges held by the peptide.

| Peptide                  | RT (min) | Theoretical mass |
|-------------------------|----------|------------------|
| Neurokinin A HKTDSFVGLM-amide | 14.2     | 1133             |
| Neurokinin A 9-11 LM-amide     | 9.2      | 261.9            |
| Neurokinin A 1-9 HKTDSFVG    | 9.4      | 893.9            |
and K2 antigens from K562 membranes within the insoluble fraction after treatment of the membranes with a Brij-Triton X-100 mixture (data not shown). However, the same antigens were evidenced, whereas the Kell K2-specific activity was enriched by nearly 50% as does a 20 °C block that prevents exit from the TGN. This observed decrease in activity not only reveals that neo-synthesized Kell K2 is inactive within these compartments, as does the observed decrease in activity following treatments with brefeldin A and monensin, both blockers of trans-Golgi network (TGN). The ensemble of these results suggest that the Kell protein, which is responsible for the lack of enzymatic activity of this blood group protein, a process closely linked to intracellular trafficking mechanisms. Thus the ensemble of the results presented show that the T193M mutation, which alters the conformation and post-translational processing of the Kell K1 antigen, does not modify its trafficking, although it abrogates all of the enzymatic activity of the protein. Interestingly, sequence alignment of NEP and the Kell protein reveals that almost all the antigenic point mutations identified thus far involve residues lying outside the active site as inferred from the known NEP x-ray structure (16), bringing to light the importance of these residues, and of the three-dimensional structure of proteins as a whole. Localization of Thr193 on the Kell K2 protein model shows this residue to sit on top of lobe 2, which is hypothesized to constitute a molecular sieve (16, 19). The introduction of a conformational change within this region by the T193M mutation could thus directly affect entry and binding of the substrate or inhibitor within the active site of the Kell K1 antigen. Hence, although the most apparent difference between Kell K1 and Kell K2 antigens lies in the disappearance of a glycosylation in Kell K1, our results show that it is the T193M mutation that induces a conformational change in the Kell protein, which is responsible for the lack of enzymatic activity of the K1 antigen of Kell.

Although questions regarding other Kell antigens are now open, the Kell K2 protein is herein unambiguously revealed as a metalloprotease part of the M13 family. Indeed, the identification of a small fluorogenic model substrate allowed the pharmacological profile of this antigen to be drawn. Hence, Kell K2 is inhibited by increasing zinc concentrations and, in accordance with its cell surface localization, active at a physiological pH (7–8). Moreover, it is inhibited in a highly specific manner by the generic compound phosphoramidon as well as by thiorphan, originally synthesized as a NEP-specific inhibitor but shown here to bind and inhibit Kell K2 with an equal affinity than NEP (38, 40). Compound I, a derivative of thiorphan with the generic compound phosphoramidon as well as by thiorphan, originally synthesized as a NEP-specific inhibitor but shown here to bind and inhibit Kell K2 with an equal affinity than NEP (38, 40). Compound I, a derivative of thiorphan with a biphenyl P′ moiety, bound Kell K2 with higher affinity than thiorphan with potencies reminiscent of those of NEP. Omapatrilat, a mixed inhibitor binding both NEP and ACE with high affinity and also recently shown to inhibit NEP2 activity, bound and inhibited Kell K2 with comparable affinity (Table I), whereas captopril, an ACE-specific inhibitor (52), did not bind Kell K2. This inhibitory profile closely resembles that of NEP (Table I), although Kell is its most distant member of the M13 family, the closest homologue of NEP, NEP2, shares over 60% sequence identity with NEP, it binds thiorphan and compound I with affinities 30 and 160 times lower than NEP, respectively (17), whereas these compounds are shown herein to bind Kell K2 with affinities comparable to those of NEP. Hence despite their low percentage of homology, the active sites of NEP and Kell K2 appear to be highly homologous.

Furthermore, the pharmacological profile of Kell is quite different from that of ECE-1, which has been shown to be

### Table II

| Peptide | Sequence and site of cleavage |
|---------|-----------------------------|
| Suc-AAF-AMC | Suc–AA ↓ F–AMC |
| Glut–FAF-AMC | Glut–FAA ↓ F–AMC |
| Aβ 1–40 | DAFEEHDGYEVQIKVVFADVGSA |
| Bradykinin | RPQGPSFR |
| Met-Enkaphalin | YGGFM |
| Angiotensin I | DRSYIFPPL |
| ANP | TAPRSLRRSSCFGGRMDRIGAQSGLGCNSFRY |
| Neurotensin | PLYQNKPRRPYIL |
| GnRH | pHNSGLRPQ–NH2 |
| α-MSH | Acetyl–SYSMHEFHKWKPV |
| Oxytocin | CTIQNCPLG |
| Gastrin | RPQGLMEDAYGNNFD |
| ACTH | SYSEHEFHKWPVKKRPPVKVYPVNA |
Enzymatic Activity of Kell K1 and Kell K2 Antigens

insensitive toward thiorphan and to bind phosphoramidon with an affinity in the micromolar range (as opposed to nanomolar concentrations for NEP, NEP2, and Kell) (7). Although previously published data have suggested that Kell K2 is not inhibited by phosphoramidon with high affinity (38), this study used an engineered, secreted soluble form of the protein (sKell) and produced the recombinant enzyme by infection of insect cells, which may not constitute an appropriate reflection of the physiological form of Kell expressed in mammalian systems, as indicated, for instance, by its complete inactivity at a physiological pH on one hand and by its only partial inhibition at (very) high concentrations of phosphoramidon on the other hand (38). Nevertheless, this secreted form of Kell K2 had been shown to efficiently cleave Big-endothelins, precursors to the vasoconstrictive endothelin peptides. This action of the Kell K2 antigen is confirmed herein where it is shown to efficiently cleave Big-endothelins, precursors to the vasoconstrictive endothelin peptides. This action of the Kell K2 antigen is confirmed herein where it is shown to efficiently cleave Big-endothelins, precursors to the vasoconstrictive endothelin peptides. This action of the Kell K2 antigen is confirmed herein where it is shown to efficiently cleave Big-endothelins, precursors to the vasoconstrictive endothelin peptides. This action of the Kell K2 antigen is confirmed herein where it is shown to efficiently cleave Big-endothelins, precursors to the vasoconstrictive endothelin peptides. This action of the Kell K2 antigen is confirmed herein where it is shown to efficiently cleave Big-endothelins, precursors to the vasoconstrictive endothelin peptides. This action of the Kell K2 antigen is confirmed herein where it is shown to efficiently cleave}

However, this will necessitate further exploration of the Kell active site and the design of powerful and specific inhibitory compounds. Finally, the inactive Kell K1 antigen expressed in 9% of the population may constitute a potentially novel risk factor in multifactorial diseases involving messenger peptide homeostasis. However, the impact of the expression of this antigen on the substrates defined herein will need to be explored in vivo.

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Taken together, the ensemble of these results reveals the very unique characteristics of the Kell proteins. Similar to its homologues of the M13 family, the substrate specificity of the Kell K2 protein relies upon the recognition of the P1 and P2 amino acids, which are preferentially bulky hydrophobic residues. Although it cleaves small synthetic fluorogenic peptides, it was more specific in its cleavage than NEP or NEP2 (28). However, Kell K2 does not display a preference for small messenger peptides, as has been observed for NEP. Hence contrary to NEP, which does not cleave messenger peptides bigger than ~3 kDa like the big endothelins, Kell K2 binds and cleaves these messenger peptide precursors with high affinity, providing this metalloprotease with an NEP-like pharmacological profile but an ECE-like substrate specificity. However, Kell K2 does not degrade the amyloid peptide Aβ1–40, which has recently been shown to be efficiently cleaved by ECE-1 and NEP in vitro and probably in vivo (18, 53), precluding any involvement of this blood group in amyloid clearance phenomena. This last result brings forward the possibility of exploring enzymatically active Kell antigens as novel therapeutic targets in the treatment of inflammatory processes and/or hypertension.
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