Topography of Kell Blood Group Protein and the Expression of Multiple Antigens by Transfected Cells

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Kell is one of the major blood group systems in human red blood cells (RBCs). The Kell antigens are carried on a 731 amino acid glycoprotein that is thought to span the erythrocyte membrane once. Rabbit antibodies to three synthetic peptides, derived from different parts of the Kell protein, were used to determine the topology of Kell protein on the RBC. Antibodies to a C-terminal peptide and to a peptide derived from amino acid residues 410 to 439 reacted with RBCs treated with 0.2 mol/L dithiothreitol. An antibody to the N-terminal peptide reacted with inside-out RBC vesicles but not with right-side-out vesicles nor with intact RBCs, showing that Kell is a type II membrane protein and that the extracellular portion of the protein is folded by disulfide bonds. By transfection, Kell protein was expressed on the cell surface of surrogate cells, and the transfected cells expressed similar antigenic properties as native RBCs. Kell protein was expressed in COS-1 and K562 cells and in Sf9 cells infected by the Baculovirus system. Transfected K562 cells expressed several high-incidence antigens but not the low-incidence antigen K1.

THE KELL BLOOD GROUP system consists of a large number of antigens that may be organized in five antithetical sets with opposing high- and low-incidence antigens. Kell antigens are destroyed by chemicals that disrupt disulfide bonds, suggesting that the alloimmune antibodies react with conformational epitopes on folded Kell proteins. All human alloimmune antibodies tested immunoprecipitated a surface-exposed glycoprotein that, based on reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), is about 93 to 97 kDa. In nonreducing conditions, larger protein complexes in excess of 115 kDa are noted, which may be due to the association of Kell with other protein components of the red blood cell (RBC).

Based on the partial amino acid sequence of a protein immunoprecipitated with two antibodies against high-incidence Kell antigens, K7 or K14, we cloned a cDNA from a human bone marrow (BM) library. The cDNA encoded a 731 amino acid glycoprotein with a single putative membrane-spanning region. Based on charge differences on either side of the membrane-spanning region and on the locations of N-glycosylation sites, it was postulated that Kell is a type II protein that spans the membrane once, has a short, 46 amino acid, N-terminal cytoplasmic domain, and has a large, 665 amino acid, C-terminal portion exposed to the outer surface.

In this study, we confirm the topology of Kell protein in RBC membranes and show that transfected cells express Kell protein on the cell surface with similar antigenic properties as native RBCs.

MATERIALS AND METHODS

Antibodies. Mouse monoclonal antibodies (MoAbs) to K2 and K14 were gifts from Dr Pablo Rubinstein (New York Blood Center, New York, NY), and antibody to K1 was a gift from Dr D. Goossens (CNRS, Paris, France). Human alloimmune antibodies to various Kell antigens are from the Laboratory of Immunohematology (New York Blood Center). Rabbit antibodies were developed to three synthetic peptides derived from amino acid sequences in different locations on the Kell protein. The N-terminal peptide corresponds to amino acids 2 to 31, another peptide (no. 410) comprises amino acids 410 to 439, and the C-terminal peptide corresponds to the 30 amino acids at the C-terminal end. Antiserum to Kell peptides were purified by affinity chromatography using the appropriate peptide bound to Sepharose 4B (Pharmacia, Uppsala, Sweden).

Flow cytometry analysis. RBCs, or transfected cells, were suspended in phosphate-buffered saline (PBS) with or without dithiothreitol (DTT), incubated for 10 minutes at room temperature, and washed twice with PBS containing 5% fetal calf serum (FCS). The cells were incubated on ice for 30 minutes with the appropriate antibody. Transfected cells were then fixed for 15 minutes at room temperature in PBS containing 2% formaldehyde. After 2 washes with PBS/FCS, a 1:50 dilution of fluorescein-conjugated second antibody was added for 30 minutes. Analysis was performed with a FACScan (Becton Dickinson Instruments, Mountain View, CA).

Transient expression in COS cells. A 2.2-kb cDNA containing the entire coding region for Kell protein was inserted into an expression vector pBCCI21 being HindIII and Xba I cloning sites. COS-1 cells were transfected by the calcium phosphate precipitation method. Expression of recombinant Kell protein was measured after 48 hours of transfection. The expression vectors used and the culture conditions have been previously described.

Expression in stable-transfected K562 cells. K562 cells grown in suspension culture in RPMI medium with 10% FCS were cotransfected with pBCCI21 containing Kell cDNA as described above and with pRSV-Neo. Stable-transfected cells were selected by resistance to 0.4 mg/mL geneticin. Mock-transfected cells were transfected only with pRSV-Neo.

Induction of K562 cells with hemin. Stable mock-transfected cells and cells transfected with expression vector containing Kell cDNA were incubated with 20 mmol/L hemin (Sigma, St Louis, MO) for 2 days in 850-cm² tissue culture roller bottles (Falcon; Becton Dickinson). This treatment induces the synthesis of hemoglobin and some RBC membrane antigens. Expression of K14 antigens on the cell surface was determined by fluorescence-activated cell sorter (FACS) analysis at the start of the experiment (day 0) and after 2 days of incubation with hemin.

Expression in the Baculovirus system. Kell cDNA was subcloned into the Baculovirus transfer vector (PV L 1393; Invitrogen Corp, San Diego, CA) using Xba I and Bgl II sites. Sf9 cells maintained in suspension culture (TNM-FH media) were co-transfected with wild-type viral DNA and with the recombinant transfer vector. The recombinant virus was selected by visual screening, was puri-
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Fig 1. Extracellular location of the C-terminal peptide and of amino acid residues 410 to 439. Untreated RBCs and cells treated with 0.2 mol/L DTT were incubated with rabbit antibodies to either the C-terminal peptide or to peptide no. 410 and binding was measured by FACS analysis. (A) RBCs of common Kell phenotype (K) or Ko(null) RBCs were treated with antibody to the C-terminal peptide. (B) Binding of antibody to peptide no. 410 to RBCs of common Kell phenotype. (B) Absence of DTT; (B) with 200 mmol/L DTT.

RESULTS

Antibodies to the C-terminal peptide and to peptide no. 410 bind to the outer surface of RBCs. As determined by FACS analysis, antibodies to the C-terminal peptide and to peptide no. 410 reacted weakly with intact RBCs of normal Kell phenotype not treated with reducing reagents. On treatment with 200 mmol/L DTT there was a fivefold to sevenfold increase in binding of antibody (Fig 1A and B). The effect of DTT was noted at concentrations as low as 12.5 mmol/L, and the effect increased linearly up to 200 mmol/L (data not shown). Only background levels of antibody-binding were noted with Ko(null) RBCs that do not express any Kell-related surface antigens. DTT, up to 200 mmol/L, did not increase the binding of antibodies to Ko(null) cells (Fig 1A).

N-terminal portion of Kell protein is on the inside surface of RBCs. Right-side-out and inside-out RBC vesicles were treated with antibody to the N-terminal peptide, and surface binding was measured by FACS analysis. There was very little, if any, binding of antibody to right-side-out vesicles, but the antibody reacted with inside-out vesicles, indicating that the N-terminal segment of Kell protein is expressed to the inner surface of the membrane (Fig 2).

Further evidence was obtained by surface-labeling intact RBCs with [35S]methionine for 2 hours as described for another system. Cells were extracted with 0.5% sodium deoxycholate and 1% Triton X-100 (Sigma) and were centrifuged for 15 minutes at 12,000 rpm in a microfuge to remove insoluble material. The supernatant fraction was diluted threefold with PBS, and, after overnight incubation at 4°C with rabbit antibody to the C-terminal peptide, protein A Sepharose was added for 2 hours at room temperature to isolate the immune complex. The proteins were separated by 7.5% SDS-PAGE and were detected by autoradiography.

Surface-labeling of RBCs and preparation of inside-out and right-side-out RBC membrane vesicles. RBCs were surface-labeled with [35S]methionine for 2 hours. Detergent-soluble extracts were immunoprecipitated with antibody to the C-terminal peptide, and proteins were separated by SDS-PAGE and detected by autoradiography. There was a greater yield of right-side-out than of inside-out vesicles, as shown by the larger amount of surface-labeled proteins (Fig 3A, lane 2). Radioactive Kell protein was immunoprecipitated only from inside-out vesicles (Fig 3B, lane 1) and not from right-side-out vesicles (Fig 3A, lane 1).

Expression of Kell protein by COS-1 cells, K562 cells, and S9 cells. Transiently transfected COS-1 cells and stable-transfected K562 cells were metabolically labeled with [35S]methionine for 2 hours. Detergent-soluble extracts were immunoprecipitated with antibody to the C-terminal peptide, and proteins were separated by SDS-PAGE and detected by autoradiography. Both COS-1 (Fig 4A) and K562 cells (Fig 4B) expressed a 93-kD radioactive protein that was immunoprecipitated with the antibody. This radioactive protein, which is of the same apparent molecular weight as Kell protein isolated from RBCs, was not present in mock-transfected cells (Fig 4).

In the Baculovirus system, infected SF9 cells, but not non-infected cells, expressed a protein that, by SDS-PAGE and Western immunoblotting using antibody to the C-terminal peptide, was recognized as recombinant Kell protein (data not shown).

Recombinant Kell protein expresses a Kell surface antigen
Antibody to N-Terminal peptide

Fig 2. Antibody to the N-terminal peptide binds to the surface of inside-out but not right-side-out vesicles. Right-side-out (RO) and inside-out (IO) RBC vesicles were incubated with rabbit antibody to the N-terminal peptide and binding was measured by FACS analysis. Solid bars (I) show the binding to right-side-out vesicles, and the cross-hatched bars (II) are to inside-out RBC vesicles.

(K14). Noninfected and infected Sf9 cells and mock-transfected and stable-transfected K562 cells were analyzed for K14 surface antigens using a specific MoAb to K14. K14 is a high incidence Kell antigen present in over 99% of all persons. Similar to other Kell antigens, treatment of RBCs with 200 mmol/L DTT inactivates the K14 antigen, suggesting that presentation of the epitope requires a disulfide-bonded protein. As determined by FACS analysis, only background amounts of anti-K14 was bound to mock-transfected K562 and to noninfected Sf9 cells. Infected Sf9 cells and stable-transfected K562 cells bound fivefold to tenfold more antibody than the control cells (Fig 5). Because K14 is a naturally occurring Kell antigen, these results indicate that transfected K562 cells and infected Sf9 cells are able to insert Kell protein in the plasma membrane in a sufficiently folded manner to mimic the expression of this naturally occurring Kell epitope. As noted with RBCs, treatment of transfected cells expressing Kell protein with 200 mmol/L DTT inhibited the binding of anti-K14.

Treatment of K562 cells with hemin. To determine whether hemin-induced proteins enhance the expression of a recombinant Kell surface-antigen, mock-transfected and transfected K562 cells were treated with hemin, and surface-expression was measured by FACS analysis using a MoAb to K14. After 2 days of treatment with hemin, mock-transfected K562 cells showed an increased (twofold) binding of monoclonal anti-K14 (Fig 6A). As noted earlier, stable-Kell-transfected K562 cells, before hemin treatment, expressed several-fold more K14 epitope on the surface than did mock-transfected cells (Fig 5). Treatment of the stable-Kell-transfected cells with hemin did not further increase the expression of recombinant Kell antigens (Fig 6B). However, hemin appeared to sharpen the FACS profile suggesting that not all geneticin-selected cells were expressing recombinant Kell protein, and treatment with hemin induced Kell expression only in those cells that were not expressing recombinant Kell.

Transfected K562 cells express high-incidence but not low-incidence antigens. K2 and K14 are high-incidence Kell antigens noted in over 99% of the population; by contrast, K1 is only found in about 9%. K1 has an antithetical relationship with K2 antigen. As determined by FACS analysis using MoAbs, the recombinant Kell protein expressed in K562 cells carries the high-incidence K2 and K14 antigens but does not express K1 antigen (Fig 7). This shows that these two high-incidence antigens are carried on a single Kell protein.

In contrast to MoAbs, alloimmune sera give a high background of nonspecific binding with both COS-1 and K562 cells. Nevertheless, in transfected K562 cells, there was noticeable greater binding of antibody to the high-incidence antigens K2, K5, and K7 but not to the low-incidence antigens K1 and K3 (data not shown).

Fig 3. Kell protein is immuneprecipitated by antibody to the N-terminal peptide from inside-out but not right-side-out RBC vesicles. RBCs were surface-labeled with 3H and inside-out and right-side-out vesicles prepared. The vesicles were treated with antibody to the N-terminal peptide, the immune complex was separated by SDS-PAGE, and the radioactive proteins were detected by autoradiography. Proteins from right-side-out vesicles (A) and from inside-out vesicles (B) are shown. Lane 1 has immune-precipitated proteins, and lane 2 has the total surface-labeled proteins.
membrane protein with a short, 46 amino acid, hydrophilic N-terminal cytoplasmic domain and a large, 665 amino acid C-terminal domain exposed to the cell surface. Kell protein contains 16 cysteine residues, with 1 of them present in the membrane-spanning region and the other 15 cysteines in the extracellular portion of the protein. The cysteine residues occur in 2 clusters just outside of the membrane-spanning region and near the C-terminal end. The large number of cysteine residues suggests extensive folding by disulfide interactions. This is in keeping with early biochemical studies that showed inactivation of Kell antigens by reducing reagents. The present studies on the topology of Kell protein using antibodies directed to the N-terminal or C-terminal segments of Kell protein confirm our proposal. The antibodies to the C-terminal portion, or to amino acid residues 410 to 439, react on the outer surface of RBCs, if the RBCs have been reduced with DTT. This shows the extracellular location of these segments of the Kell protein and also indicates that Kell protein is folded by disulfide bonds. These results further suggest that the C-terminal segment and amino acid residues 410 to 439 are only exposed to the antibodies when the protein is unraveled by treatment with DTT. Evidence that the N-terminal segment is in the cytoplasmic side of RBCs was obtained by showing that antibody to this domain reacts with inside-out vesicles and not with right-side-out membrane vesicles. A diagram showing the topology of Kell protein and the locations of the peptides to which antibodies were prepared is shown in Fig 8.

DISCUSSION

Hydropathy analysis of the amino acid sequence encoded by a Kell cDNA obtained from a BM library indicated that Kell protein has a single membrane-spanning region. Based on the charge differences on either side of the membrane-spanning region and the location of possible N-linked glycosylation sites, we proposed that Kell protein is a type II

Fig 4. Expression of Kell protein by COS-1 and K562 cells. Transiently transfected COS-1 and stable-transfected K562 cells were incubated with L-[35S]methionine for 2 hours at 37°C. The radiolabeled proteins were immunoprecipitated from a detergent-soluble fraction with rabbit antibody to the C-terminal peptide. The immune complex was separated by SDS-PAGE, and the radioactive proteins were detected by autoradiography. COS-1 cells (A) and K562 cells (B) are shown. Lane 1 has the immunoprecipitated proteins from cells transfected with a vector containing Kell cDNA, and lane 2 from mock-transfected cells.

Fig 5. K14 is present on the surface of K562 and Sf9 cells expressing Kell protein. Mock-transfected and transfected K562 cells and noninfected and infected Sf9 cells were analyzed for K14 surface antigens by FACS analysis. An MoAb to K14 was used. K562 cells (A) and Sf9 cells (B) are shown. The black outline indicates increased binding of anti-K14 to the surface of K562 cells and Sf9 cells expressing Kell protein.
However, it is possible that other erythroid proteins are needed for full expression of Kell antigens, because serologic studies have indicated that full expression necessitates the presence on RBCs of Kx antigen. McLeod RBCs, which lack Kx antigen, are characterized by weak expression of Kell antigens. Reciprocally, Ko(null) RBCs that do not express Kell antigens have enhanced Kx activity. Kx is not the only antigen thought to be associated with Kell. The Leach phenotype (which lacks glycoporphin C and D) and the Gerbich phenotype (which has an altered form of glycoporphin D) show weak expression of Kell antigens. It is not known whether K562 cells express Kx antigens, but treatment with hemin did not further increase the surface-expression of recombinant Kell-related antigens, indicating that hemin-induced proteins are not involved in enhancing
Kell antigen expression. Hemin treatment of K562 cells is known to increase the expression of erythroid markers such as globin and some blood group antigens, but hemin also increases the synthesis of other proteins, including a heat-shock protein.

We do not know the Kell phenotype of the person from whom the BM library was prepared for the isolation of Kell cDNA. However, subsequent studies in our laboratory have shown that a person with a common Kell phenotype has mRNA that encodes for a protein of identical protein sequence (data not shown), indicating that the original BM cDNA library was prepared from tissues of common Kell phenotype. The transfection studies show that a single expressed protein carries more than one Kell antigen. Two high-incidence antigens K2 and K14 were expressed. However, the recombinant protein does not express the low-incidence antigen K1. The fact that more than one antigen is carried on a single protein is consistent with the studies of Petty et al and Parsons et al that showed, using immunological techniques, that Kell blood group antigens occur as clusters on Kell glycoprotein. We do not yet know, at the molecular level, what modifications to the protein determines the expression of these low-incidence antigens, but it is known that low-incidence antigens such as K1, K3, and K6 are also expressed by a protein encoded by the same gene and carried by a protein similar in size. There are several examples in the glycoporins in which single amino acid changes cause blood group antigen polymorphisms. A similar mechanism may exist in the Kell blood group system.

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