Human Erythrocyte Antigens: II. The In(Lu) Gene Regulates Expression of an Antigen on an 80-Kilodalton Protein of Human Erythrocytes

By Marilyn J. Telen, Thomas J. Palker, and Barton F. Haynes

THE In(Lu) GENE is the only dominant autosomal gene recognized to inhibit the expression of erythrocyte alloantigens encoded for by independently segregating genes. Although first described as an inhibitor of expression of Lutheran antigens, the In(Lu) gene was later shown to down-regulate expression of the P, i, and Auberger (Au') erythrocyte antigens. Although the P, and i antigens are polysaccharides whose structures are fairly well understood, the biochemical nature of Lutheran antigens has remained undefined. The In(Lu) gene could affect the expression of several unrelated erythrocyte blood group antigens by encoding for a carbohydrate transferase that blocks subsequent addition of carbohydrate moieties required for various antigens to exist. However, no evidence has heretofore been uncovered regarding the target molecules on which the In(Lu) gene product might act. Thus, in this study, using a monoclonal antibody (A3D8) previously found to identify an erythrocyte antigen inhibited by the In(Lu) gene, we have immunoprecipitated proteins of 80 and 170 kD from normal Lu(b+) erythrocytes. We have further shown that the A3D8 antigen resides on the 80-kD protein, and that, in contrast to Lu(b+) erythrocytes, this 80-kD protein is immunoprecipitated in trace amounts from In(Lu) Lu(a−b−) erythrocytes, whereas the 170-kD protein is present in normal amounts on both Lu(b+) and In(Lu) Lu(a−b−) erythrocytes.

MATERIALS AND METHODS

Production of Monoclonal Antibody

The A3D8 murine hybridoma cell line was established by fusion of BALB/c spleen cells from animals immunized with malignant Sézary T cells with the P3 X 63/Ag8 BALB/c myeloma cell line. The A3D8 cell line was cloned and maintained as previously described and produces a monoclonal antibody whose heavy chain is IgM. AE-1 and AE-2 hybridoma cell lines, which produce antierythrocyte acetylcholinesterase antibodies, were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). DMH 1.335 hybridoma monoclonal antibody was a gift of Nicole Bernard and Bernard Amos (Duke University, NC). This antibody reacts with the human Ia-like antigen, does not react with human erythrocytes, and therefore was used as an IgM negative control.

Assays to Measure Cell Surface A3D8 Antibody Reactivity Before and After Enzymatic Degradation

Cell surface A3D8 antibody reactivity was measured by radioimmunoassay (RIA) as previously described. Briefly, erythrocyte suspensions (5% vol/vol) were incubated 30 minutes at 25 °C with dilutions of A3D8 ascitic fluid or control IgM murine myeloma protein in triplicate or quadruplicate wells of V-bottom microtiter plates (Dynatech, Alexandria, Va.). Erythrocytes were washed three times in Dulbecco's phosphate-buffered saline (DPBS) with 1.0% bovine serum albumin (BSA), 0.1% gelatin, and 0.1% sodium azide. Erythrocytes were then resuspended in DPBS, and 125I-labeled affinity-purified F(ab')2 anti-mouse Ig antibodies (Amersham Corp., Arlington Heights, Ill.) were used to detect specific binding of A3D8 antibody. RIA data were expressed as dcpm, as determined by the equation: 

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\Delta\text{cpm} = \text{mean cpm experimental} - \text{mean cpm control.}
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For analysis of the effect of chymotrypsin, erythrocytes were treated with chymotrypsin (Type II, Sigma Chemical Co., St. Louis) for 30 minutes at 37 °C, washed three times with RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), and assayed as described above. Erythrocytes were treated with bromelin (Bromelase, Dade Division, American Hospital Supply Corp., Miami) according to manufacturer's directions, ie, 15 minutes at 25 °C, and then washed and assayed as above. Erythrocytes were treated with 2-aminoethylisothiouronium bromide (AET) by modification of the procedure described by Sirich and colleagues to produce cells with a paroxysmal nocturnal hemoglobinuria-like membrane defect. Briefly, fresh cells collected in ethylenediamine...
tetraacetic acid (EDTA) were washed three times in PBS. Eight-percent AET was made in 10 mmol/L phosphate, pH 8.0. Packed cells (0.5 mL) were incubated with 2 mL AET solution for nine minutes at 37 °C. Control cells were incubated in PBS, pH 8.0, for an equal amount of time at 37 °C. All cells were then washed in PBS five times, or until all supernatants were clear. AET treatment always caused visible hemolysis. Cells were then brought to 5% vol/vol suspensions for use in radioimmunoassay (RIA).

Radioiodination of Erythrocytes and Peripheral Blood Leukocytes

Erythrocytes were obtained from heparinized or acid citrate dextrose-preserved whole blood by separation through PBS, 5 mmol/L EDTA, 1% dextran (T500, Pharmacia, Sweden) for 30 to 60 minutes at 4 °C. After removal of the leukocyte compartment and dextrose-preserved whole blood by separation through PBS, 5 minutes at 37 °C. Control cells were incubated in PBS, pH 8.0, for 60 minutes at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C. SAC was pelleted, and the supernatant was then incubated (SAC, Calbiochem-Behring Corp. La Jolla, Calif) for one hour at 4 °C.
of AET treatment abrogated erythrocyte agglutination by both anti-Cellano \textsuperscript{18} and anti-Lutheran b antisera, as well as reactivity by RIA with murine monoclonal antiacetylcholinesterase antibodies AE-1 and AE-2. \textsuperscript{19} Thus, these data demonstrate that A3D8 antigen activity on erythrocytes is sensitive to inactivation by a variety of proteases, namely, trypsin, chymotrypsin, and bromelin, as well as by the sulfhydryl-containing reagent AET.

**Immunoprecipitation of the Erythrocyte A3D8 Antigen**

Multiple immunoprecipitations from \textsuperscript{125}I-labeled erythrocytes of blood group O or A donors yielded a major band of approximately 80,000 mol wt, as detected by SDS-PAGE under reducing conditions (Fig 1). Two other bands of mol wt 50,000 and 30,000 were also present under reducing conditions (Fig 1). The ability to detect these bands and their apparent molecular weights did not vary with cells from either Lu(a-b+) or Lu(a+b+) donors. Analysis under non-reducing conditions of the A3D8 antigen immunoprecipitated from \textsuperscript{125}I-labeled erythrocytes also yielded a major band of approximately 80,000 mol wt. However, a second band, corresponding to approximately 170,000 daltons, was seen under nonreducing conditions (Fig 1). The 50,000- and 30,000-dalton bands were not seen under nonreducing conditions. When immunoprecipitated proteins were analyzed under reducing and nonreducing conditions on the same gel, the nonreduced band at about 80 kD appeared to have migrated slightly faster than the reduced 80-kD band (Figs 1 and 2). In some instances, a faint band was also visualized just above the 80-kD band; this band appeared to be a nonspecific contaminant, most likely consisting of band 3 protein, which was also seen to a lesser extent with immunoprecipitation with control antibody (Figs 1B and 2A).

Immunoprecipitations from \textsuperscript{125}I-labeled peripheral blood mononuclear cells also yielded a major 80-kD band when electrophoresed under reducing conditions (data not shown).

Immunoprecipitations from \textsuperscript{125}I-labeled erythrocytes of the original In(Lu) Lu(a−b−) proposita (M.C.)\textsuperscript{1} showed strikingly different results from those seen with Lu(b+) cells. Under nonreducing conditions, electrophoresis of immunoprecipitated protein from these cells produced a predominant 170,000-dalton protein and a trace 80,000-dalton band (Fig 2), the reverse of the findings using Lu(b+) cells. When electrophoresed under reducing conditions, the proteins immunoprecipitated from the In(Lu) Lu(a−b−) cells appeared as heavy 50,000- and 30,000-dalton protein bands and a faint 80,000-dalton protein band (Fig 2).
Again, these results were the reverse of those obtained using Lu(b+) cells.

Two-dimensional gel analysis of the immunoprecipitated proteins from Lu(b+) cells showed that upon reduction, the 170,000-dalton protein appeared to be composed of 50- and 30-kD subunits, whereas the 80-kD protein showed only a streaking effect, consistent with a heavily glycosylated 80-kD protein (Fig 3A). Similar analysis of In(Lu) Lu(a–b−) cells showed that the 170-kD protein of In(Lu) cells was also composed of 50- and 30-kD subunits (Fig 3B).

Detection of A3D8 Antigen by Western Blot Technique

A3D8 antibody bound to a single protein band of approximately 80 kD when Lu(b+) erythrocyte membrane proteins were first separated by SDS-PAGE under nonreducing conditions, transferred electrophoretically to nitrocellulose paper, incubated with A3D8 antibody, and counterstained with 125I-F(ab')2 anti-mouse antibody in the Western blot technique (Fig 4). No 170-kD band was seen. However, when membrane proteins were separated under reducing conditions, no A3D8 antibody bound to protein transferred to nitrocellulose (data not shown).

When erythrocyte membrane proteins from In(Lu) Lu(a–b−) erythrocytes were used, A3D8 bound extremely faintly in the 80-kD region. Again, no antibody was bound in the 170-kD region (data not shown).

DISCUSSION

We have previously shown that the A3D8 monoclonal antibody recognizes an antigen whose expression on erythrocytes, on a subpopulation of mononuclear leukocytes, and in plasma is down-regulated by the In(Lu) gene. We have now used enzymatic digestion, immunoprecipitation, SDS-PAGE, and Western blot techniques to show that this antigen resides on an 80-kD membrane protein of erythrocytes and leukocytes. The immunoprecipitation of a second protein of 170 kD from normal cells and in an apparently increased amount from In(Lu) cells, along with lack of detectable antigen activity on a similar molecular weight protein in the Western blot system, is a puzzling finding. Our data do not rule out the possibility that the antigen exists on both proteins and is irreversibly denatured by SDS on the 170-kD protein, thus preventing identification of the antigen at 170 kD in the Western blot system. However, since we have shown that total A3D8 antibody binding to In(Lu) cells is reduced to about 20% of that using Lu(b+) cells, the total amount of A3D8 antigen on In(Lu)
Fig 3. Two-dimensional gel analysis of immunoprecipitates obtained with A3D8 antibody from Lu(b+) and In(Lu) Lu(a−b−) erythrocytes. A3D8 and control IgM antibodies were used in duplicate immunoprecipitation experiments, as described in Materials and Methods. Immediately after completion of electrophoresis under nonreducing conditions in the first dimension, one of the two duplicate lanes containing the immunoprecipitate obtained with A3D8 antibody was incubated for 30 minutes in buffer containing 5% 2-mercaptoethanol (reducing conditions). This lane was then inserted into a second slab gel apparatus and reelectrophoresed. (A) The results of one experiment using Lu(b+) cells; (B) the results obtained when In(Lu) Lu(a−b−) erythrocytes were used. In each case, the 80-kD protein showed no breakdown into lower molecular weight species upon reduction, whereas the 170-kD protein showed significant deviation from the diagonal and breakdown into two components, with approximate mol wt of 50 and 30 kD.

Fig 4. Detection of A3D8 antigen activity with Western blotting. Ghosts of Lu(b+) erythrocytes were made in hypotonic phosphate, and ghost membrane proteins were solubilized in 100 mmol/L Tris, 2% SDS, 15% glycerol. Eighty micrograms of membrane protein per lane was then electrophoresed through a discontinuous SDS-PAGE and transferred to nitrocellulose paper, as described in Materials and Methods. Lanes of nitrocellulose paper were then separated and stained with A3D8 antibody (lane A), control IgM antibody (lane B), and a hybridoma antibody that reacts with all glycophorin species (lane C), using a 125I-labeled anti-mouse Ig second antibody. A3D8 antibody identified only a band of approximately 80,000 daltons, which appeared to migrate just below PAS 1 (glycophorin alpha dimer).

In(Lu) cells must be less than on normal cells, despite the relative increase in 170-kD protein. Quantitation of the 170- and 80-kD proteins immunoprecipitated cannot be done accurately from the data presented for several reasons. First, less than 0.1% of total cpm is precipitated from normal or In(Lu) cells by A3D8 antibody in our system, despite the use of antibody in saturating amounts. Second, control antibody routinely precipitated close to 90% of cpm precipitated by A3D8, despite the significant differences in results after electrophoresis and autoradiography. Thus, cpm precipitated predominantly represented background cpm, and comparison of cpm per band would be difficult because of the low ratio of specific cpm precipitated to background cpm. Therefore, although In(Lu) cells may have more of the 170-kD protein, the increased relative density of the 170-kD band obtained from In(Lu) cells could have other explanations, such as an increased efficiency of labeling (as might be seen with decreased sialic acid content). It is also possible that the 170-kD protein may exist as a complex with the 80-kD protein but may not carry the A3D8 antigen. The association of a high molecular weight protein with one of lower molecular weight has been described for a number of cell membrane protein receptors, such as the insulin receptor.20 It is possible that the 170-kD protein acts as a receptor for the 80-kD protein. Likewise, if the 80-kD protein is a cell surface enzyme, the 170-kD protein could be a closely
linked receptor for the substrate. Although the paradoxical increase in the 170-kD protein band from \textit{ln(Lu)} cells makes this latter possibility less likely, variation in the stoichiometry of the association between the two proteins or in the efficiency of radiolabeling of the proteins could provide an explanation.

Immunoprecipitation and Western blot analysis of membrane extracts from \textit{ln(Lu)} Lu(a-b-)/ erythrocytes suggest that the amount of 80-kD protein carrying the A3D8 antigen is reduced to far less than 50% of normal, a finding that is in agreement with previously described antibody-binding data.\textsuperscript{7} If the epitope recognized by the A3D8 antibody is reduced in amount on \textit{ln(Lu)} erythrocytes, then the amount of 80-kD protein on \textit{ln(Lu)} erythrocytes would appear to be diminished by our methods, even if the protein itself existed in normal amount without the specific epitope. However, it is also possible that the \textit{ln(Lu)} gene prevents synthesis of this protein or alters it in a manner that prevents its insertion into the erythrocyte cell membrane.

There are few examples of genetic systems in which one copy of an abnormal gene suppresses expression of a normal gene product to far less than 50% of the normal level. In the common form of hereditary angioedema (HAE), one abnormal gene causes near complete absence of C1 esterase inhibitor, although the small amount of detectable C1 esterase inhibitor appears to be normal.\textsuperscript{21} It has been hypothesized, but not proved, that the dominant gene causing HAE prevents synthesis of normal protein by interfering with the regulation of transcription of the relevant DNA.\textsuperscript{21} Such a mechanism would also explain our findings. A second possibility is that the \textit{ln(Lu)} gene codes for an abnormal gene product. An abnormal gene product may interfere with the expression of the normal gene product by direct interaction with it; for example, certain variant hemoglobin chains cause accelerated degradation of the normal chains with which they associate.\textsuperscript{22} Or, as has been hypothesized for variant HAE, in which only abnormal inactive C1 esterase inhibitor is detectable, the abnormal gene product may interfere with regulation of transcription of the normal gene.\textsuperscript{21} A third possibility, and one that had been suggested prior to these studies,\textsuperscript{5} is that the \textit{ln(Lu)} gene codes for a carbohydrate transferase whose particular action prevents subsequent expression of a number of carbohydrate antigens, possibly including the Lutheran antigens as well as i and P\textsubscript{1}. Although not strictly analogous, because the \textit{H} and \textit{Se} genes are not allelic, the action of the \textit{H} gene in the presence of an \textit{Se} gene provides an example whereby action of a fucosyl transferase produces the Le\textsuperscript{a} antigen and abolishes expression of the Le\textsuperscript{b} antigen, despite the presence and simultaneous operation of the \textit{Le} gene, which would otherwise cause expression of the Le\textsuperscript{a} antigen. Studies are currently underway to produce polyclonal antisera to the 80-kD protein in order determine whether the entire protein or only the epitope recognized by A3D8 antibody is reduced.

Analysis of immunoprecipitates under nonreducing conditions on SDS-PAGE showed slightly faster migration of the 80-kD band than under reducing conditions. This, along with our inability to show antigen activity in the 80-kD region when Western blotting was performed under reducing conditions, is most consistent with a molecule that has intrachain disulfide bonds. Further evidence that these intrachain disulfide bonds exist and are necessary for expression of the A3D8 antigen is provided by the experiments using erythrocytes pretreated with AET. AET is a sulfhydryl-containing reagent that has been well documented by others to disrupt disulfide bonds necessary for both expression of certain antigen systems and activity of erythrocyte acetylcholinesterase.\textsuperscript{10,19,21} AET disruption of A3D8 antigen activity on intact cells, therefore, is strong evidence that intact disulfide bonds are required for A3D8 antigen activity.

The presence of the 50- and 30-kD bands under reducing conditions only, along with the two-dimensional gel data, is most consistent with the hypothesis that the 170-kD protein is composed of several 50- and 30-kD subunits. Western blot analysis showed, however, that the 170-kD protein need not be intact for antigen activity to be present on the 80-kD protein, since these two moieties were separated by gel electrophoresis before blotting. Therefore, reduction of the 170-kD protein into its 50- and 30-kD subunits appeared not to be responsible for loss of antigen activity by the 80-kD protein under reducing conditions.

On gels in which lysates of whole erythrocytes were run parallel to immunoprecipitates, it was clear that the A3D8 protein migrated faster than band 3 (data not shown) and glycoporphin dimer (Fig 4).

A number of serum and erythrocyte membrane proteins are approximately 80,000 daltons, including acetylcholinesterase and 5\textsuperscript{'}-nucleotidase. Acetylcholinesterase has a tissue distribution similar to A3D8 antigen activity. Acetylcholinesterase enzyme activity, like A3D8 antigen activity, has also been shown to be reduced or abolished by trypsin, chymotrypsin, bromelin,\textsuperscript{23} and AET,\textsuperscript{10} but not by neuraminidase.\textsuperscript{24} Erythrocyte acetylcholinesterase has been previously described to have a molecular weight of 75,000 to 80,000 daltons when sized by SDS-PAGE under reducing conditions, and to exist only as a 150- to 160-kD dimer under nonreducing conditions.\textsuperscript{9,25} To demonstrate that A3D8 did or did not bind to acetylcholinesterase, we
obtained two murine hybridoma cell lines (AE-1, AE-2) previously described to produce antierythrocyte acetylcholinesterase antibodies. In parallel immunoprecipitation experiments, A3D8 precipitated a protein of approximately 80,000 daltons, while AE-1 and AE-2 precipitated a protein of 70,000 daltons (data not shown). Thus, it seems clear that the molecule reactive with A3D8 is not identical to that which binds to the AE-1 and AE-2 antibodies, and therefore, that A3D8 probably does not bind to the acetylcholinesterase species identified by these antibodies.

Another question regarding the protein identified by A3D8 is whether or not Lutheran antigens reside on it. Lutheran antigens have been previously shown to be sensitive to a variety of proteolytic enzymes, including trypsin, chymotrypsin, and bromelin, and to be resistant to bacterial neuraminidase. Although not abrogated by 6% AET treatment as originally described, Lutheran antigens were made nonreactive by the 8% AET treatment that we have used to remove A3D8 reactivity and antiacetylcholinesterase antibody reactivity. Therefore, the A3D8 protein could be the backbone onto which the Lutheran antigens are added.

It also is of note that the A3D8 protein is similar in tissue distribution and molecular weight to a murine protein that has been shown to be involved in organ-specific homing of lymphocytes to nodal high endothelial venules. This murine protein is on medullary thymocytes, circulating B and T lymphocytes, and erythrocytes, thus showing the same tissue distribution as A3D8 antigen. However, immunoprecipitation of murine lymphocyte membrane proteins with a monoclonal antibody to this antigen does not also precipitate a higher molecular weight species under nonreducing conditions. We have shown that A3D8 antibody neither produces nor blocks blastogenesis of lymphocytes, but the function of the A3D8 protein on human lymphocytes remains to be delineated. The A3D8 protein is also similar in tissue distribution and maps to the same chromosome as the antigen identified by antibody F 10.44.2. This latter antigen is also on nearly all circulating leukocytes, as well as on erythrocytes, on brain tissue, and in plasma. Like A3D8, it also binds primarily to the medullary population of thymocytes. However, whether the antigen recognized by F 10.44.2 undergoes suppression of expression by the In(Lu) gene is not known.

Thus, we have shown that the In(Lu) gene regulates expression of an antigen residing on an 80,000-dalton protein of erythrocytes and leukocytes. Moreover, action of the In(Lu) gene causes variation in the amounts of 80- and 170-kD protein precipitable with the A3D8 monoclonal antibody but does not cause the appearance of a protein that migrates anomalously on SDS-PAGE analysis. The 80- and 170-kD proteins appear to be distinct from other previously described erythrocyte membrane proteins. The identification of these proteins should help elucidate further the mechanism of action of the In(Lu) gene and perhaps the nature of the Lutheran antigens.

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MJ Telen, TJ Palker and BF Haynes