Isolation of the JMH Antigen on a Novel Phosphatidylinositol-Linked Human Membrane Protein

By Kristie A. Bobolis, John J. Moulds, and Marilyn J. Telen

JMH is a high-frequency human erythrocyte blood group antigen. Previous work has shown that JMH is absent from complement-sensitive erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH); such cells have a broad defect in expression of phosphatidylinositol (PI)-linked proteins. Using both human JMH antisera and a JMH-like murine monoclonal antibody, we have identified a 76-Kd membrane protein present in JMH-positive but not JMH-negative erythrocytes. A similar 76-Kd JMH protein was also identified on a human lymphoid T-cell line, HS-2. Using PI-specific phospholipase C, a small amount of JMH antigen could be cleaved from intact erythrocytes and immunoprecipitated from the supernatant of treated erythrocytes, thus confirming that the protein bearing the JMH antigen is anchored by a PI-linkage to the erythrocyte membrane. This protein was further shown not to be identical to decay accelerating factor (70 Kd), a previously identified PI-anchored protein of somewhat similar molecular weight.

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MATERIALS AND METHODS

Antibodies to JMH. Human antisera containing antibodies to the high-frequency blood group antigen JMH were obtained from the Reference Laboratory of Gamma Biologicals, Inc (Houston, TX). Several human JMH antisera were tested, and all gave similar results. One serum, Meged, has been described previously by Moulds et al as one of several antisera that appear to subdivide the JMH blood group.9 This serum was used in most immunochemical experiments presented in this study because of the availability of relatively large quantities of this serum and because it contained antibody in high titer. Unless otherwise specified, anti-JMH refers to the antibody in this serum.

The murine monoclonal antibody (MoAb) H8, which has been shown to have JMH-like, or more specifically, Meged-like reactivity, was provided by Dr Robert Knowles (Sloan-Kettering, New York, NY).24 This antibody was produced by immunizing female Balb/c mice with a human lymphoid T-cell line, HS-2, originally derived from a patient with acute lymphocytic leukemia.21

All human antisera and control nonreactive human sera were first purified by adsorption onto JMH-positive RBCs and then eluted from intact RBCs using a rapid acid elution technique (Gamma-ELU KIT II; Gamma Biologicals). This step reduced nonspecific protein binding in immunoprecipitation and immunoblotting experiments, compared with ones in which human serum was not processed in this manner.

Cells. Erythrocytes used were routinely collected in tubes containing EDTA, washed repeatedly in phosphate-buffered saline (PBS), and stored in Alsever's solution at 4°C. Cells used for biochemical assays were drawn the same day of use. Rare JMH-negative erythrocytes were obtained from a member of the family with autosomal dominant inheritance of the JMH-negative phenotype, as described by Kollmar et al.8 In this family, no hematologic abnormality has been noted in affected family members.

Antibodies to PI-linked membrane proteins. Rabbit antiserum to DAF (CD55) was produced as previously described9 and provided by Dr Wendell Rosse (Durham, NC). Anti-lymphocyte function-associated antigen-3 (LFA-3; CD58) MoAb was provided by Dr Timothy Springer (Boston, MA).20 The cell line producing antiacetycholinesterase MoAb AE-224 was obtained from the American...
Type Culture Collection (Rockville, MD). MoAb 2/24D4 to erythrocyte membrane inhibitor of reactive lysis (MIRL; CD59) was submitted to the MABS 1990 workshop (Lund, Sweden) by Dr Anne Fletcher and was also used in this study. Anti-DAF MoAb 3.3-136-41 was provided by Dr Robert Knowles.

Radioimmunoassays (RIAs) of the expression of JMH and PI-linked proteins. Antibody reactivity with random donor as well as phenotypically JMH-negative erythrocytes was measured by RIA as previously described. Three lymphoid cell lines, HSB-2 T cells, HUT 78 T cells, and SB B cells, as well as PBL from normal donors were also assayed for expression of JMH antigen. Binding of rabbit antisera was detected using radiolabeled Staphylococcal protein A (SPA); reactivity with murine MoAbs was detected with 

Radioimmunoassays of JMH antigen. 1575

Radioimmunoprecipitation of JMH antigen after PI-specific phospholipase C (PI-PLC) treatment of erythrocytes. Normal erythrocytes were radiolabeled as previously described. Five hundred-microliter aliquots of radiolabeled cells were incubated for 45 minutes at 37°C with either 10 µL (0.095 U) of PI-PLC (isolated from *Bacillus thuringiensis*; ICN Biomedicals, Cleveland, OH) or 10 µL of PBS. The samples were then centrifuged at 12,000g for 5 minutes and the supernatants were recovered and stored at 4°C. The cells were then washed in PBS, incubated with antibody at 37°C for 60 minutes, and processed as described previously. The supernatants were preclarified with 300 µL of RIF buffer containing 10% SPA for 4 hours at 4°C and then centrifuged at 12,000g for 5 minutes. The supernatants were then incubated with antibody for 4 hours at 4°C, precipitated with goat antimouse or antihuman IgG chromatography gel overnight, and further processed as described for RBC lysates.

In some experiments, RBCs were incubated with PI-PLC or PBS in the presence of the following protease inhibitors: phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Company, St Louis, MO) 100 µg/mL, Leupeptin (Sigma) 5 µg/mL, and Pepstatin A (Sigma) 5 µg/mL. The RBCs and supernatants were then collected and processed as above.

RESULTS

RIA of leukocytes, normal erythrocytes, and JMH-negative erythrocytes. RBCs from normal donors and from a donor with the autosomal dominant JMH-negative phenotype were tested by RIA for expression of known PI-linked proteins and JMH protein. As shown in Table 1, the JMH-negative erythrocytes demonstrated absent or greatly reduced binding with human anti-JMH sera and no binding with murine monoclonal H8 antibody. Two examples of JMH-negative erythrocytes were available for assay of expression of PI-linked proteins, and showed normal binding of antibodies to these proteins, including MoAbs to MIRL (CD59), which showed 94% binding compared with the mean of normal control erythrocytes. JMH-negative erythrocytes bound 85% to 118% as much monoclonal and polyclonal antibodies to DAF (CD55) as did normal cells,

<table>
<thead>
<tr>
<th>Specific cpm Bound</th>
<th>JMH+</th>
<th>JMH+</th>
<th>JMH+</th>
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<td>638</td>
<td>537</td>
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<td>2,408</td>
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Abbreviation: NT, not tested.

**Normal JMH+ erythrocytes from different donors.

**Murine MoAbs to JMH.
91% to 97% as much monoclonal anti-LFA-3 (CD58), and 83% to 106% as much antiacetylcholinesterase as compared with normal controls.

Murine monoclonal H8 antibody to JMH showed weak reactivity with PBL from a normal donor and weak reactivity with lymphoid cell lines HSB-2 T cells, SB B cells, and HUT 78 T cells, as previously described. Leukocytes from JMH-negative donors were not available for testing.

The number of copies of JMH antigen expressed on JMH-positive erythrocytes could not be directly ascertained, as anti-JMH antisera were not available in quantities sufficient for purification and direct radiolabeling. However, the number of cpm per erythrocyte observed in RIAs was comparable with that obtained with antigens expressed at 1,000 to 3,000 copies per cell.

Radioimmunoprecipitation of erythrocyte JMH protein. Immunoprecipitation from random donor 125I-radiolabeled erythrocytes yielded a band of approximately 76 Kd when human anti-JMH eluate was used as precipitating antibody (Fig 1). This protein was not identified when nonreactive eluate from nonimmune human serum was used instead of anti-JMH serum eluate. When MoAb H8 was used as precipitating antibody, a protein of similar molecular weight (76 Kd) was identified (Fig 1). This protein was not present when nonimmune myeloma protein P3 was used instead of H8. The apparent molecular weight of this protein showed little or no change when analyzed under nonreducing and reducing conditions (Fig 1).

We also performed simultaneous parallel immunoprecipitation experiments using erythrocytes from a normal individual and from an individual with the autosomal dominant JMH-negative phenotype. Again, a protein of approximately 76 Kd was identified using human anti-JMH or murine monoclonal H8 on normal RBCs. This 76-Kd band was absent from JMH-negative erythrocytes when human anti-JMH or H8 were used as precipitating antibody (Fig 2, lanes 3 and 4). However, immunoprecipitation of JMH-negative erythrocytes with human anti-JMH yielded a band at 80 to 81 Kd. This band was not identified on JMH-negative erythrocytes when H8 was used as precipitating antibody. It is not clear at this time whether the 80 to 81-Kd band identified with the anti-JMH antibody is a nonspecific contaminant (most likely consisting of dimeric glycophorin A) or whether it is a variant JMH-like protein, as adequate numbers of JMH-negative cells were not available for thorough investigation of this band. Lane 5 of Fig 2 shows the identical electrophoretic mobilities of the dimeric glycophorin A and the 80 to 81-Kd membrane protein under nonreducing conditions.
**Radioimmunoprecipitation of leukocyte JMH protein.** H8 murine MoAb to JMH protein precipitated a 72- to 74-Kd protein from radiolabeled HSB-2 T cells (Fig 3, lane 2), a human lymphoid T-cell line. JMH protein was also precipitated from HSB-2 cells using human anti-JMH antibody (not shown). This protein band was absent from precipitates obtained with nonreactive murine myeloma protein (Fig 3, lane 1).

Detection of JMH protein by Western blot technique. Immunoblots of normal hemoglobin-free erythrocyte membrane proteins first separated by SDS-PAGE under nonreducing conditions showed a protein band of 75 to 76 Kd when incubated with human anti-JMH (Fig 4, lanes 1 and 4). H8 MoAb showed a similar distinct band of 75 to 76 Kd in immunoblots of normal erythrocyte membrane proteins (not shown), as did immunoblots using a second human anti-JMH (not shown). This 76-Kd band was not shown on blots of JMH-negative erythrocytes from an individual with the autosomal dominant JMH-negative phenotype (Fig 4, lanes 2 and 3). This band was also absent when normal erythrocytes (lane 5) and JMH-negative erythrocytes (lane 6) were incubated with human serum lacking anti-JMH.

Radioimmunoprecipitation of JMH protein from PI-PLC-treated erythrocytes and solutions containing PI-PLC-released proteins. PI-linked surface membrane proteins appear to be incompletely and variably released from the cell by treatment with PI-PLC. However, treatment of erythrocyte membranes with this enzyme is still a useful method of examining cell surface proteins thought to be linked to the membrane via phosphatidylinositol. The effects of PI-PLC on erythrocyte DAF, MIRL, acetylcholinesterase, and C8 binding protein have been extensively reported by others.

After treatment of normal erythrocytes with PI-PLC, reactivity with JMH antisera was only minimally reduced. Anti-JMH antibody specifically precipitated a protein of 76 Kd from radiolabeled treated cells (Fig 5, lane A1), although in lesser amount than from non-PI-PLC–treated erythrocytes (Fig 5, lane B1). Most importantly, however, human anti-JMH also specifically precipitated a protein of 76 Kd from the supernate of the PI-PLC–treated RBCs, but not from the supernate of the buffer-treated RBCs (Fig 5). In addition, however, anti-JMH specifically precipitated another protein that appeared to be spontaneously released at 37°C. This protein was immunoprecipitated from the supernates of both PI-PLC–treated and buffer-treated erythrocytes and had a molecular weight of 67 Kd (Fig 5); it was not precipitated with nonreactive human serum. Similar results were obtained using H8 MoAb in place of human anti-JMH.

Further investigation of the results of PI-PLC treatment showed no obvious differences in the radiolabeled proteins of the erythrocytes incubated with PI-PLC compared with those of the cells incubated with buffer. There was also no apparent differences between the supernates of erythrocytes incubated with PI-PLC compared with buffer-treated cells, also as previously described.

To ensure that PI-linked proteins were being specifically released during treatment with PI-PLC, anti-DAF MoAb 3.3-141-36 was used in parallel immunoprecipitation studies, along with MoAbs to two non–PI-linked proteins, glycophorin A and p80 (CD44). As expected, the PI-linked protein DAF was shown in the supernate of PI-PLC–treated cells, but not in the supernate of buffer-treated cells (data not shown). As described before, the molecular weight of DAF released by PI-PLC was found to be slightly less (by ~3 Kd) than membrane-associated DAF, which has a molecular weight of ~70 Kd (not shown). In addition, immunoprecipitation with anti-p80 and antiglycophorin A showed that these non–PI-linked proteins were not released into the supernates of PI-PLC– or buffer-treated cells (not shown).

Effect of protease inhibition on JMH protein released from PI-PLC-treated erythrocytes. Anti-JMH antibody specifically precipitated a 76-Kd protein from PI-PLC–treated erythrocytes and from the supernates of PI-PLC–treated cells both in the absence (Fig 6, lanes A1 and A2) and presence of protease inhibitors (Fig 6, lanes B1 and B2, heavy arrow). However, when radiolabeled RBCs were treated with PI-PLC enzyme or enzyme buffer in the presence of protease inhibitors, the lower molecular weight protein of 67 Kd (Fig 6, lane A2, light arrow) was not precipitated by anti-JMH antibody from the supernate of PI-PLC–treated cells (Fig 6, lane B2) or buffer-treated cells (not shown).

Comparison of JMH protein with DAF. When hemoglobin-free ghosts were prepared from 125I-surface–labeled normal erythrocytes and used in immunoprecipitation experiments with monoclonal anti-DAF 3.3-141-36, human anti-JMH, and H8 MoAb, anti-DAF specifically precipitated a protein with an apparent molecular weight of 69 Kd. In contrast, human anti-JMH and H8 precipitated a protein.
Fig 4. Western blot of JMH+ and JMH− erythrocytes. Human anti-JMH identified a protein band of 76 Kd in lanes 1 and 4, containing membrane proteins from JMH+ erythrocytes. This band was not visualized in lanes 2 and 3, containing proteins from JMH− erythrocytes. Lane 5 contains proteins from JMH+ erythrocytes and lane 6 contains proteins from JMH− erythrocytes stained with nonreactive human serum.

DISCUSSION

Evidence presented here shows that the protein bearing the high frequency erythrocyte antigen JMH is a novel PI-linked protein. In radioimmunoprecipitation studies with human antisera to JMH and H8 MoAb, a 76-Kd protein was isolated from normal erythrocytes, but not from JMH-negative erythrocytes (Fig 2), which serologic data have suggested are missing all JMH-related antigens. The molecular weight of this protein showed little or no change when analyzed under nonreducing and reducing conditions, indicating that intrachain disulfide bonds do not greatly influence the size of the protein, despite the fact that reagents that destroy such bonds are known to prevent serologic detection of JMH antigens. Immunoblots of normal and JMH-negative erythrocyte membrane proteins separated by SDS-PAGE under nonreducing conditions also showed reactivity of H8 and human anti-JMH antibodies with a 76-Kd protein present only in JMH-positive cell membranes. Radioimmunoprecipitation studies of the T-cell line HSB-2 also showed that a similar JMH protein is expressed by leukocytes, although the molecular weight of the leukocyte protein may be slightly lower. Both human anti-JMH and MoAb H8 precipitated a 72- to 74-Kd JMH protein from radiolabeled HSB-2 cell membranes.

JMH protein appears distinct from numerous other PI-linked erythrocyte membrane proteins, as JMH-negative erythrocytes expressed normal amounts of CD55, CD58, CD59, and acetylcholinesterase. Further immunochemical studies showed JMH protein to differ from the 70-Kd CD55, and preliminary results also suggest that JMH protein is not the 65- to 68-Kd PI-linked complement regulatory protein designated C8-binding protein or homologous restriction factor (data not shown).

The ability of purified PI-PLC from B thuringiensis to
ISOLATION OF JMH ANTIGEN

There appears to be no appreciable change in electrophoretic mobility of JMH released into supernate after incubation with PI-PLC compared with membrane-bound JMH (Fig 5, lane A3). Studies with other PI-anchored proteins released with PI-PLC have shown variable results. DAF19,22 and LFA-310 show more rapid electrophoretic migration after PI-PLC cleavage, whereas MIRL20 appears to have slightly decreased migration after PI-PLC cleavage. The mobility of another PI-linked protein, the Fcγ receptor (CD16) of PB mononuclear cells, appears also to be unaffected by treatment with PI-PLC.25

In addition to the 76-Kd form of JMH specifically released by PI-PLC treatment of normal erythrocytes (Fig 5, lane A3, and Fig 6, lanes A2 and B2), JMH antisera appeared to specifically interact with a 65- to 69-Kd protein released into supernate of cells incubated at 37°C for 45 minutes (Fig 5, lanes A3 and B3, and Fig 6, lane A2). Protease inhibitors prevented appearance of this protein in cell supernates (Fig 6, lane B2). This suggests that some form or portion of JMH protein is released from the RBC membrane during incubation at 37°C, likely due to the action of an endogenous RBC protease.

There has been great interest in proteins linked to the membrane through a PI anchor. These functionally diverse proteins are well discussed elsewhere.4,5,18,26,27 It has been speculated that the PI anchor might provide proteins at least two novel and perhaps functionally important properties in some cases.4,18,27,28 First, the anchor of PI-linked cell surface proteins is located within the outer leaflet of the bilayer, allowing relatively easy lateral mobility of proteins.7,30 Integral membrane proteins, on the other hand, are anchored by a hydrophobic sequence of amino acids and often have intracytoplasmic components that interact with the cytoskeleton, restricting lateral movement.29 Second, the existence of PI-specific phospholipases that re-

Fig 6. PI-PLC treatment of erythrocytes in the presence of protease inhibitors. Radiolabeled erythrocytes were treated with PI-PLC in the presence (B) or absence (A) of protease inhibitors. The cells and supernates containing released membrane proteins were analyzed for the presence of JMH protein. Lanes A1 and B1 (heavy arrow) show JMH protein precipitated with anti-JMH antibody from membrane lysates. Lanes A2 and B2 (heavy arrow) also show JMH protein precipitated with anti-JMH antibody from the supernates of PI-PLC-treated cells in the absence and presence of protease inhibitors, respectively. The supernates of enzyme-treated cells in the absence of protease inhibitors also yielded a specific 67-Kd protein with anti-JMH antibody (lane A2, light arrow). The supernates of buffer-treated cells also yielded this band in the absence of protease inhibitors (data not shown). This protein was not precipitated from the supernates of enzyme-treated cells (lane B2) or buffer-treated cells (data not shown) in the presence of protease inhibitors. Neither band was isolated from the supernates of PI-PLC-treated cells using control reagents (lanes A3 and B3).

cleave a portion of the JMH protein from normal erythrocyte cell surfaces provides further evidence that JMH is anchored to the plasma membrane by covalently attached PI. The fact that only a portion of JMH was released by PI-PLC is consistent with what is seen with several other PI-anchored proteins on human erythrocytes, including LFA-3,19 DAF,25 MIRL,26 and acetylcholinesterase.24 Resistance of certain human PI-linked proteins to cleavage by PI-PLC from bacterial or protozoan sources has been attributed to structural modifications of the basic PI-anchor that influence susceptibility to PI-PLC.4 The structural basis for this resistance in the case of human erythrocyte acetylcholinesterase is known to be due to palmitoylation of the inositol ring.24

Fig 7. Differentiation of JMH protein from DAF (CD55). An autoradiograph of a gel containing JMH protein immunoprecipitated by human and murine antibodies (lanes 1 and 2, respectively) and DAF precipitated by MoAb 3.3-141-36 (lane 3) clearly illustrates that the two proteins exhibit distinct relative mobilities. Lane 4 contains precipitate obtained with a nonreactive human reagent.

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lease PI-linked proteins from membrane surfaces by hydrolyzing the PI anchor has led to speculation that a function of the anchor might be to mediate the selective release of certain surface membrane proteins. While this is an intriguing possibility, there is no clear evidence, at least in mammalian systems, that PI cleavage occurs as an event that controls interaction at the cell surface.\textsuperscript{,27,21}

In conclusion, evidence is provided in this study that a novel PI-linked protein of 76 Kd bears the JMH blood group antigen. This is now the second family of blood group determinants shown to be associated with a PI-linked protein, as we have shown previously that DAF (CD55) bears the Cromer blood group antigen.\textsuperscript{,27,21} While the anchor might be to mediate the selective release of PI-linked proteins from membrane surfaces by hydrolyzing the PI anchor has led to speculation that a function of the JMH-negative phenotype has not been associated with hematologic abnormalities, although an acquired JMH-negative phenotype has been found in elderly patients with rheumatologic diseases or lymphoma. We have recently identified a family with congenital hemolytic anemia of unclear etiology; the JMH protein is weakly expressed by affected family members, while other PI-linked proteins are normally expressed. However, erythrocytes from affected family members do not show increased sensitivity to complement-mediated lysis.\textsuperscript{31} These data suggest that the JMH protein may be functionally important or that the JMH gene may be located near another gene important to RBC integrity. A more detailed understanding of the biochemistry and distribution of JMH may provide further insight into possible functions of this protein.

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