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Immunology

Sequence of the Lyb-2 B-cell differentiation antigen defines a gene superfamily of receptors with inverted membrane orientation

(cDNA clones/CD23/asialoglycoprotein receptor)

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ABSTRACT **Lyb-2 is a mouse B-cell differentiation antigen expressed on the surface of pre-B cells and B cells but not on terminally differentiated antibody-secreting plasma cells. Lyb-2 has been shown to play a role in B-cell activation and differentiation and may be a receptor for a B-cell growth factor or lymphokine. We have isolated and sequenced cDNA encoding the Lyb-2.1 allele. Lyb-2 mRNA is expressed only in B-lineage cells and is absent from antibody-secreting cell lines. The predicted protein contains 354 amino acids and is lacking an amino-terminal signal peptide. The protein is shown to be oriented with its carboxyl terminus external to the cell. Sequence comparisons demonstrate that Lyb-2 is homologous to the asialoglycoprotein receptor and to CD23, the B-cell-specific Fc receptor for IgE, both of which are oriented with their carboxyl termini external to the cell. These molecules, therefore, constitute a gene superfamily of cell surface receptors with inverted membrane orientation.**

Lyb-2 is a 45-kDa mouse cell surface glycoprotein whose expression appears limited to cells of the B-lymphocyte lineage (1–3). It has been shown to be expressed on B cells but not T cells in spleen, on cells in bone marrow, lymph node, and fetal liver, and on both pre-B and B-cell lymphoma lines (1, 3). However, Lyb-2 expression is lost upon terminal differentiation of B cells into antibody-secreting plasma cells (3, 4). It is also absent from thymus, kidney, liver, testis, and brain (1). Conventional antisera and monoclonal antibodies (mAbs) have identified at least three allelic forms of Lyb-2 with distinct peptide maps: Lyb-2.1, -2.2, and -2.3 (3, 5). Classical genetic studies indicate that Lyb-2 is encoded by a single genetic locus on mouse chromosome 4 (6, 7).

Although the precise function of Lyb-2 in B-cell differentiation and/or activation is not known, a variety of data suggest that it may be a receptor for a B-cell growth factor or lymphokine. Two mAbs specific for Lyb-2.1 have been shown to inhibit the antibody response by splenic B cells to T-cell-dependent antigens but not to T-cell-independent antigens (8, 9). A factor other than interleukin 2 in the culture supernatant of Con A-activated spleen cells could prevent the inhibition of the antibody response to sheep red blood cells induced by anti-Lyb-2.1 (9). One mAb specific for Lyb-2.1 (10.1.D2) has been shown to directly transform a fraction of small splenic B cells into blasts and to induce B-cell proliferation in the presence or absence of T cells (9–11). A second anti-Lyb-2.1 mAb (9-6.1) does not by itself stimulate B cells to divide, but synergizes with culture supernatants containing B-cell growth factor activity from two T-cell lines (12). Anti-Lyb-2 also induces B-cell expression of Ia antigens (13, 14) and mobilizes cytoplasmic free Ca^{2+} in purified small splenic B cells (14). Yakura *et al.* (12) suggested that Lyb-2 might either be the receptor or closely associated with the receptor for B-cell-stimulatory factor 1 (interleukin 4). However, other studies indicate that Lyb-2 is clearly distinct from the interleukin-4 receptor in size and cell distribution (15) as well as functional properties (13, 14).

To further define the function of Lyb-2, we have isolated and sequenced cDNA clones* encoding this protein. We find that Lyb-2 mRNA is expressed in pre-B cells and B cells, but not in antibody-secreting cells or non-B cells. We demonstrate that Lyb-2 has an inverted orientation with respect to the cell membrane in that its carboxyl terminus is located outside the cell. The predicted amino acid sequence of Lyb-2 is homologous to those of two receptor molecules that have been shown to have a similar inverted orientation: the asialoglycoprotein receptor (ASGPR) and CD23, the B-cell-specific Fc receptor for IgE. These molecules, therefore, define a gene superfamily of receptors with an inverted membrane orientation.

MATERIALS AND METHODS

Cell Lines and mAbs. L1.2 is an Abelson murine leukemia virus-transformed pre-B-cell line established from bone marrow cells of an adult C57L mouse and was a gift of I. Weissman (Stanford University). 70Z/3 is a chemically induced pre-B-cell line from a $(C57BL/6 \times DBA/2)F_1$ mouse and was obtained from I. Weissman. BCL_1 (clone CW13.20-3B3) is a spontaneous B-cell lymphoma line from a BALB/c mouse and was obtained from the American Type Culture Collection (ATCC). This clone does not secrete immunoglobulin (although IgM secretion can be induced). 38C13 is a carcinogen-induced B-cell lymphoma line from a C3H mouse, and V6 is a subclone (S. Levy, Stanford University). It may secrete extremely low levels of immunoglobulin. J558 and MOPC-315 are IgA-secreting myeloma cell lines of BALB/c mouse origin and were obtained from the ATCC. Cy34.1.2 is a mouse hybridoma cell line that secretes IgG1 mAb (specific for the mouse B-cell differentiation antigen Lyb-8.2) and was obtained from the ATCC. VL3/1 is a C57BL/Ka mouse thymoma cell line (from I. Weissman). HT-2 is an interleukin-2-dependent mouse T-cell line and was grown in the presence of recombinant human interleukin 2 from Cetus. Pre-B cells from BALB/c mouse bone marrow were cultured according to Whitlock and Witte (16) using the bone marrow-derived stromal cell line AC6.21 (17) (gift of I. Weissman).

Hybridoma cells producing anti-Lyb-2.1 mAb 10.1.D2 (IgG2b) were a gift of D. Mosier (Medical Biology Institute, La Jolla, CA). The mAb was purified from the culture supernatant of these cells on a protein A column. Anti-Lyb-2.1 mAb 9-6.1 (IgG2b) was purchased from New England Nuclear.

cDNA Library Construction and Screening. RNA was isolated from the L1.2 pre-B-cell line by the guanidine thiocyanate/CsCl method (18). Poly(A)$^+$ RNA was selected using oligo(dT)-cellulose (type 3, Collaborative Research) column chromatog-

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Abbreviations: mAb, monoclonal antibody; ASGPR, asialoglycoprotein receptor.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04170).

raphy. A cDNA library was constructed according to Okayama and Berg (19) using 4 μg of poly(A)$^+$ RNA from L1.2, the pcDV1 vector primer, and the pL1 linker fragment. Approximately 4 × 10^5 independent colonies were obtained after transformation into competent DH5α bacterial cells. DNA and frozen bacterial stocks were prepared from a 1-liter culture of the initial transformed bacterial cells. Size-fractionated sublibraries were then constructed from the primary library. DNA (30 μg) from the primary library was digested with either *Sfi* I or *Cla* I (each of which linearizes the vector). The DNA was electrophoresed on a 1% agarose gel. DNA fractions corresponding to insert sizes of 1–2 kilobases (kb) and to >2 kb were extracted separately, religated, and transformed into competent DH5α cells. DNA and frozen stocks were prepared from 1-liter cultures of these sublibraries.

DNA from the two size-selected cDNA sublibraries was transfected into COS-7 cells by using DEAE-dextran (20). COS-7 cells transiently expressing Lyb-2.1 were selected by "panning" according to Seed and Aruffo (20), using a combination of two anti-Lyb-2.1 mAbs, 10.1.D2 and 9-6.1. Plasmids from selected COS-7 cells were recovered by the procedure of Hirt (21) and retransformed into competent DH5α cells. Two further cycles of transfection by spheroplast fusion (22), selection by panning, recovery, and transformation of plasmid DNA were performed. Eleven of the resulting bacterial colonies were randomly chosen for analysis.

Immunofluorescence Analysis. Transfected (DEAE-dextran) COS-7 cells were stained for Lyb-2.1 48 hr after transfection. Transfected cells or untransfected cells were stained with 250 ng of protein A-purified mAb 10.1.D2 and/or 2 μg of ascites fluid containing mAb 9-6.1 per 10^6 cells, followed by fluorescein-conjugated goat anti-mouse antibodies (Tago). Immunofluorescence was determined by analysis on a modified FACS II (Becton Dickinson) fluorescence-activated cell sorter.

Nucleotide Sequence. The insert of pcDVLyb2.1-7 and its smaller restriction fragments were subcloned into M13mp18 and -mp19 (23) and sequenced fully in both directions by using the dideoxy chain-termination method (24).

Northern Blotting. RNA was isolated from cell lines and tissues by using the guanidine thiocyanate/CsCl method (18). Poly(A)$^+$ RNA from 38C13 subclone V6 was provided by S. Levy (Stanford University). Northern blotting and hybridization were performed as described (25) except the high temperature washes were at 65°C. The hybridization probe was an 800-base-pair (bp) *Sac* I–*Bam*HI fragment of the insert of cDNA clone pcDVLyb2.1-7 (containing protein coding sequence from amino acid 75 to amino acid 350) labeled with ^{32}P by random hexamer priming (26).

Immunoprecipitation. pcDVLyb2.1-7 DNA was transfected into COS-7 cells by using the DEAE-dextran method (20). After 48 hr the cells were detached using 0.5 mM EDTA/0.02% sodium azide in isotonic phosphate-buffered saline and an aliquot was shown to stain positively for Lyb-2.1 on the fluorescence-activated cell sorter. Approximately 3.6 × 10^6 transfected COS-7 cells, 5 × 10^6 untransfected COS-7 cells, and 2 × 10^7 L1.2 cells were surface-iodinated with 0.5 mCi of Na^{125}I (1 Ci = 37 GBq; Amersham) with lactoperoxidase (27). Cells were washed in phosphate-buffered saline and lysed in 0.5 ml of lysis buffer (1% Nonidet P-40/10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.15 M NaCl/1 mM phenylmethylsulfonyl fluoride). Lysates were precleared with normal mouse serum and protein A-Sepharose (Pharmacia) prior to specific immunoprecipitation. The L1.2 lysate was divided into two aliquots: 20% was immunoprecipitated with 2 μg of ascites fluid containing a control mAb specific for the *b* allele of mouse β_2-microglobulin (New England Nuclear), which coprecipitates associated H-2 heavy chains. The remaining 80% of the L1.2 lysate and the entire lysates of the transfected and untransfected COS-7 cells were immunoprecipitated with a combination of two mAbs specific for Lyb-2.1: 2.1 μg of protein A-purified mAb 10.1.D2 and 10 μg of ascites fluid containing mAb 9-6.1. Lysates were incubated for 2 hr at 4°C, followed by an additional 2 hr at 4°C in the presence of protein A-Sepharose. The precipitates were washed as described (25). The precipitated protein was eluted by resuspension of the protein A-Sepharose beads in 50 μl of 0.2 M sodium phosphate, pH 7.0/0.1% NaDodSO$_4$/1% 2-mercaptoethanol and boiling for 2 min. After cooling to room temperature, the supernatants were transferred and the pellets discarded. Samples were adjusted to 0.1 M sodium phosphate, pH 7.0/0.05% NaDodSO$_4$/1% Nonidet P-40/50 mM EDTA/0.05% 2-mercaptoethanol/soybean trypsin inhibitor (0.2 mg/ml) (Sigma)/protease inhibitors [0.25 μg/ml each leupeptin, antipain, pepstatin, and chymostatin (Sigma)]. Half of each sample was treated with 0.25 unit of endoglycosidase F (Boehringer Mannheim), and both the treated and untreated samples were incubated for 3 hr at 37°C. Loading buffer (28) was added to a final concentration of 1× and samples were applied to a 12% NaDodSO$_4$/polyacrylamide gel with a 6% stacking gel and electrophoresed as described (28).

RESULTS

Isolation of cDNA Clones Encoding Lyb-2.1. We constructed a cDNA library in the pcDV expression vector system (19) using mRNA from the Lyb-2.1$^+$, Abelson murine leukemia virus-transformed pre-B-cell line L1.2. This library was transfected into COS-7 cells and transient transfectants were screened for Lyb-2.1 surface expression by using a panning method (20). After three cycles of screening 11 bacterial colonies were chosen randomly for independent analysis. As shown for clone pcDVLyb2.1-7 (Fig. 1*C*), transfection of plasmid DNA from 10 of these colonies into COS-7 cells yielded positive cell surface immunofluorescence staining for Lyb-2.1 with a mixture of two anti-Lyb-2 mAbs. Plasmid DNA from the single negative colony in this expression assay (Fig. 1*B*) did not contain an insert. In contrast, all 10 positive plasmids contained inserts of approximately 1.45 kb with identical restriction maps. Transfection of a mixture of DNA from three of these plasmids yielded COS-7 cells that stained positively with each of the two anti-Lyb-2.1 mAbs individually (Fig. 1 *E* and *F*).

pcDVLyb2.1-7 Hybridizes to mRNA Specific for B Lineage Cells. On Northern blot analysis the insert of cDNA clone pcDVLyb2.1-7 hybridized to a 1.7-kb mRNA species ex-

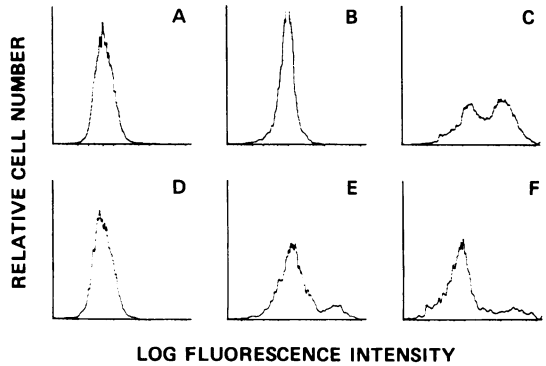

FIG. 1. Cell surface expression of Lyb-2.1 on transfected COS-7 cells. Untransfected (*A*) or transfected (*B–F*) COS-7 cells were stained with anti-Lyb-2.1 mAbs 10.1.D2 and/or 9-6.1 followed by fluorescein-conjugated goat anti-mouse antibodies as a second stage. Stained cells were analyzed on the fluorescence-activated cell sorter. (*A*) Untransfected COS-7 cells stained with 10.1.D2 and 9-6.1. (*B*) COS-7 cells transfected with clone pcDV-8 (no insert) and stained with 10.1.D2 and 9-6.1. (*C*) COS-7 cells transfected with Lyb-2.1 clone pcDVLyb2.1-7 and stained with 10.1.D2 and 9-6.1. COS-7 cells were transfected with Lyb-2.1 clones pcDVLyb2.1-1, pcDVLyb2.1-2, and pcDVLyb2.1-3 and stained with no first stage antibody (*D*), mAb 10.1.D2 (*E*), or mAb 9-6.1 (*F*).

pressed in spleen, in pre-B cells cultured from bone marrow, in the Abelson virus-transformed pre-B-cell line L1.2, in the chemically induced pre-B-cell line 70Z/3, and in the B-cell line BCL_1 (Fig. 2). Very small amounts of this same mRNA species could be detected after a long exposure in RNA from lymph node, the B-cell tumor line 38C13, thymus, and lung. It is likely that the very low expression in the latter two tissues reflects the presence of small numbers of endogenous B cells. No hybridizing mRNA was detectable in the plasma cell lines J558 and MOPC-315 (both of which secrete IgA antibodies), the antibody-secreting B-cell hybridoma line Cy34.1.2, T-cell lines HT-2 and VL3/1, liver, kidney, brain, or intestine. This pattern of mRNA expression is highly consistent with the observed pattern of expression of Lyb-2 protein. Since the selection of Lyb-2.1 cDNA clones was based upon epitope expression and not tissue specificity, the Northern blot data provide strong evidence that the isolated cDNA clones indeed encode Lyb-2.1.

Nucleotide and Predicted Amino Acid Sequence of Lyb-2.1 cDNA. The nucleotide sequence of cDNA clone pcDV-Lyb2.1-7 contains a single long open reading frame encoding a polypeptide chain of 354 amino acids beginning with an amino-terminal methionine (Fig. 3). The predicted molecular weight of this polypeptide is 40,496. The sequence contains 36 bp of 5′ untranslated region preceding the initiation codon and 310 bp of 3′ untranslated region followed by a poly(A) tail. The poly(A) tail is not preceded by a canonical poly(A) addition signal (AATAAA), although there are several related sequences in the appropriate location [e.g., AATATT or TTTAAA ending, respectively, 12 or 6 bp upstream of the poly(A) tail]. The most striking feature of the predicted amino acid sequence is the lack of an amino-terminal hydrophobic segment consistent with a signal peptide (Fig. 3). This is atypical for cell surface proteins, and clone pcDVLyb2.1-7 was isolated based on cell surface expression of the encoded

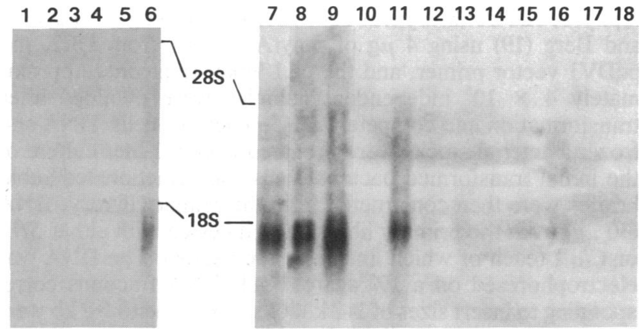

FIG. 2. Expression of Lyb-2 mRNA. A Northern blot of RNA was hybridized to a ^{32}P-labeled Lyb-2 cDNA probe. RNA samples were from the following sources. Lanes: 1, 38C13 clone V6; 2, MOPC-315; 3, J558; 4, Cy34.12; 5, HT-2; 6, B-cell culture from BALB/c bone marrow; 7, L1.2; 8, 70Z/3; 9, BCL_1; 10, VL3/1; 11, spleen; 12, lymph node; 13, thymus; 14, liver; 15, kidney; 16, lung; 17, brain; 18, intestine. All samples were total RNA except for 38C13 subclone V6, which was poly(A)$^+$-selected RNA. Twenty micrograms was loaded in each lane except for lanes 1 (5 μg), 6 (10 μg), and 7 (10 μg). rRNA markers are indicated.

protein. Examination of the sequence (Fig. 3) and a hydrophobicity blot (data not shown) indicates that the predicted protein contains only a single hydrophobic segment, extending from amino acid 96 to amino acid 116. We have examined the possibility that Lyb-2 might be linked to the cell surface by a glycolipid anchor but found that cell surface expression of protein reactive with the anti-Lyb-2.1 mAb is not diminished by treatment of cells with phosphatidylinositol-specific phospholipase C (E.N. and J.R.P., unpublished results). We, therefore, conclude that the single 21-amino acid hydrophobic region of Lyb-2.1 represents the transmembrane portion of the protein. The protein contains a total of 16 cysteine

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   1                                   MetAlaAspAlaIleThrTyrAlaAspLeuArgPheValLysValProLeuLysAsnSerAla    21
   1  TAGCTGGAAGACTGTGAAGCAGAGGCGCCCAGGGCTATGGCTGACGCTATCACGTATGCAGACCTGCGCTTTGTGAAAGTGCCCCTGAAGAACAGCGCA    99

  22  SerAsnHisLeuGlyGlnAspCysGluAlaTyrGluAspGlyGluLeuThrTyrGluAsnValGlnValSerProValProGlyGlyProProGlyLeu    54
 100  TCTAACCATCTAGGACAGGACTGTGAGGCCTATGAAGATGGGGAACTCACCTACGAGAACGTGCAAGTGTCTCCAGTCCCAGGAGGGCCACCAGGCTTG   198

  55  AlaSerProAlaLeuAlaAspLysAlaGlyValGlySerGluGlnProThrAlaThrTrpSerSerValLysSerSerAlaLeuArgGlnIleProArg    87
 199  GCTTCCCCTGCACTAGCGGACAAAGCAGGGGTCGGGTCAGAGCAACCAACTGCGACCTGGAGCTCTGTGAAGTCGTCTGCTCTCAGGCAGATTCCCCGC   297

  88  CysProThrValCysLeuGlnAsnPheLeuLeuGlyLeuLeuLeuSerCysLeuMetLeuGlyValAlaValIleCysLeuGlyValArgTyrLeuGln   120
 298  TGTCCTACGGTCTGCTTGCAAAACTTCTTGCTTGGCCTTCTCCTGTCCTGTCTGATGTTAGGGGTGGCTGTCATCTGCCTGGGAGTTCGCTATCTGCAG   396

 121  ValSerGlnGlnPheGlnGluGlyThrArgIleTrpGluAlaThrAsnSerSerLeuGlnGlnGlnLeuArgGluLysIleSerGlnLeuGlyGlnLys   153
 397  GTGTCTCAGCAGTTCCAGGAGGGGACCAGGATTTGGGAAGCCACCAATAGCAGCCTGCAGCAGCAGCTCAGGGAGAAGATAAGTCAGCTGGGGCAGAAG   495

 154  GluValGluLeuGlnGluSerGlnLysGluLeuIleSerSerGlnAspThrLeuGlnGluLysGlnArgThrHisLysAspThrGluGlnGlnLeuGln   186
 496  GAGGTGGAGCTTCAGGAGTCTCAGAAAGAGCTGATCTCGAGCCAGGACACATTACAGGAGAAGCAGAGGACTCACAAGGACACTGAGCAGCAACTACAA   594

 187  AlaCysGlnAlaGluArgAlaLysThrLysGluAsnLeuLysThrGluGluGluArgArgArgAspLeuAspGlnArgLeuThrSerThrArgGluThr   219
 595  GCCTGCCAGGCTGAGAGAGCGAAGACCAAGGAGAACCTGAAAACTGAGGAGGAGCGGAGGAGGGACCTGGACCAGAGGTTGACAAGCACGCGGGAGACA   693

 220  LeuArgArgLeuSerSerCysSerSerAspThrCysCysProCysGlyTrpIleProTyrGlnGluArgCysPheTyrIleSerHisThrLeuArgSer   252
 694  CTGAGGCGCTTGTCCTCCTGTTCATCAGACACCTGCTGTCCATGCGGATGGATTCCATATCAGGAAAGGTGCTTTTACATCTCACATACCCTCAGAAGT   792

 253  LeuGluGluSerGlnLysTyrCysThrSerLeuSerSerLysLeuAlaAlaPheAspGluProSerLysTyrTyrTyrGluValSerLeuProSerGly   285
 793  CTGGAGGAGAGCCAAAAATACTGCACATCTCTGTCCTCCAAACTGGCAGCATTCGATGAACCTTCTAAGTATTACTATGAAGTTTCTCTGCCCAGCGGC   891

 286  LeuGluGluLeuLeuAspArgSerLysSerTyrTrpIleGlnMetSerLysLysTrpArgHisAspTyrAspSerGlnSerArgTyrCysAspLysIle   318
 892  TTAGAGGAGTTGCTAGATCGTTCGAAGTCATATTGGATACAGATGAGCAAGAAGTGGAGGCATGACTATGACTCTCAAAGCCGATATTGTGACAAGATA   990

 319  LysLysTyrTyrGlnLysTrpLysArgThrPheSerGluCysAlaGluLeuHisProCysIleCysGluSerGluAlaPheArgPheProAspGlyIle   351
 991  AAAAAATATTACCAGAAGTGGAAAAGAACATTTTCTGAGTGTGCAGAGCTTCACCCCTGCATTTGTGAGTCGGAGGCTTTCAGGTTTCCTGATGGGATC  1089

 352  HisLeuAsnEnd                                                                                        354
1090  CATCTGAACTGAACCGGATACTTGAACAAGACCTTGTGACCTACATCCTTAACCTAAGGCCTGCCAATTTTTAAGACTGCTATTCCTCCAGCACTCCCT  1188

1189  CACTCTCGGGCATGCCCAGCTAAGGGATGACCTGCTGCTTGCTTGAAAGCTGCTCCAGAAACTGGACTACTCTTGGGAAGAGTAAAGAAGCCTCCAGAA  1287

1288  AAGACTTGACCTTCCTTAAATACTTCCCAAACTAGAGATGGGTCAGGGGAGGGCGCTCCGCTGAGTGGATGGATGGCCCGGAGCCAGCCAGGCAGTTTT  1386

1387  ATTGAAATATTTTTAAATACTTAAAAAAAAAAAAAAAAAA                                                           1426
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FIG. 3. Nucleotide and predicted amino acid sequence of Lyb-2.1. The numbers of the first and last amino acid (above) and nucleotide (below) in each line are indicated in the left and right margins, respectively. The hydrophobic segment predicted to span the plasma membrane is overlined. Cysteine residues are marked by an asterisk (*) and the N-linked glycosylation site is indicated by a solid circle (●). Possible signals for polyadenylation are underlined.

residues, of which 3 are in the amino-terminal domain, 2 are in the transmembrane segment, and 11 are in the carboxyl-terminal domain. The region between amino acids 226 and 260 is particularly cysteine-rich, with a total of 6 cysteine residues. Lyb-2 has the properties of a glycoprotein in that it binds to lectin (29), and the sequence predicts a single N-linked glycosylation site at amino acid 136 (Asn-Ser-Ser).

Lyb-2 Is an Inverted Protein with a Carboxyl-Terminal Extracellular Domain. The lack of a traditional amino-terminal signal peptide and the presence of a hydrophobic transmembrane segment closer to the amino terminus than the carboxyl terminus are reminiscent of findings for a group of transmembrane proteins that are unusual in that they have been shown or predicted to be oriented with their amino termini within the cytoplasm and their carboxyl termini external to the cell. The fact that the only N-linked glycosylation site predicted for Lyb-2.1 is on the carboxyl-terminal side of the putative transmembrane region further suggested that Lyb-2 may also have an inverted orientation. However, it had not been determined whether the carbohydrate attached to Lyb-2 is N-linked or O-linked. We, therefore, immunoprecipitated the Lyb-2.1 protein from COS-7 cells transiently transfected with cDNA clone pcDVLyb2.1-7 and from the L1.2 pre-B-cell line after cell surface labeling with ^{125}I. Immunoprecipitates were either treated or not treated with endoglycosidase F to remove all N-linked carbohydrate. As shown in Fig. 4, treatment with endoglycosidase F reduced the size of the immunoprecipitated protein from both cell types to a common core size of M_r ≈42,000. This correlates reasonably well with the predicted core molecular weight of 40,496 based on the amino acid sequence. The untreated protein was slightly larger in L1.2 cells (M_r, ≈45,500) as compared to COS-7 cells (M_r, ≈44,000). This is not surprising given the difference in tissue and species origin of these two cell lines. Two important conclusions can be drawn from these results. (*i*) The demonstration that Lyb-2.1 contains N-linked carbohydrate (one glycan unit in each case) implies that the portion of the protein on the carboxyl-terminal side of the transmembrane segment is indeed extracellular. (*ii*) The fact that the core proteins from transfected COS-7 cells and the L1.2 pre-B cell line comigrate provides the final evidence that the clone pcDVLyb2.1-7 encodes Lyb-2.1.

Homology of Lyb-2.1 to CD23 and ASGPRs. We compared the predicted amino acid sequence of Lyb-2.1 with all protein

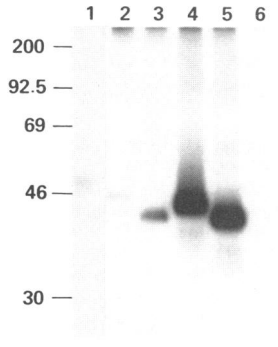

FIG. 4. Immunoprecipitation of Lyb-2.1 from a pre-B-cell line and transfected COS-7 cells. L1.2 cells (lanes 1–3), COS-7 cells transiently transfected with clone pcDVLyb2.1-7 (lanes 4 and 5), and untransfected COS-7 cells (lanes 6 and 7) were surface labeled with ^{125}I followed by immunoprecipitation from cell lysates. A mAb specific for mouse β_2-microglobulin (*b* allele) was used for lane 1, resulting in the visible band that represents co-immunoprecipitation of H-2 molecules. Anti-Lyb-2.1 mAbs 10.1.D2 plus 9-6.1 were used for lanes 2–7. Samples in lanes 3, 5, and 7 were treated with endoglycosidase F, while those in lanes 1, 2, 4, and 6 were not. All samples were electrophoresed on a 12% $NaDodSO_4$/polyacrylamide gel. The migration positions of comigrated ^{14}C-labeled molecular weight standards are shown in the left margin ($\times 10^{-3}$).

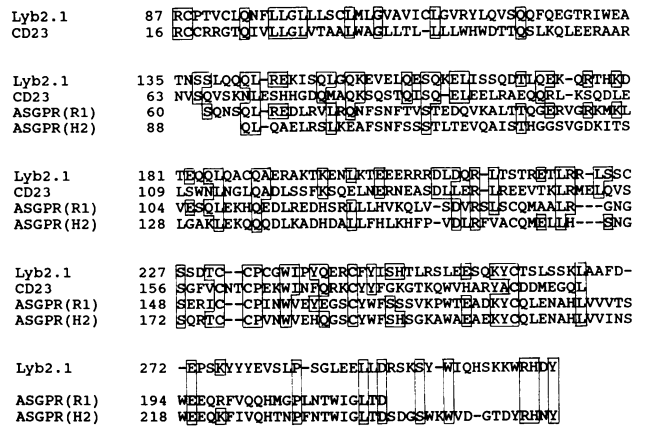

FIG. 5. Comparison of Lyb-2.1 sequence to CD23 and ASGPRs. The amino acid sequences of homologous regions of Lyb-2.1, CD23, and human (H2) and rat (R1) ASGPRs are aligned using the one-letter amino acid code. Residues identical to those in Lyb-2.1 are boxed. Gaps are indicated by dashes. The number of the first amino acid in each line is shown in the left margin. The sequences of CD23 and the ASGPRs H2 and R1 were obtained from the National Biomedical Research Foundation protein sequence database.

sequences available in the National Biomedical Research Foundation data base (June 24, 1988) using the FASTP program (30). As shown in Fig. 5, we found striking homology between the Lyb-2.1 sequence and those of human CD23 (the low-affinity Fc receptor for IgE) and both rat (R1) and human (H2) ASGPRs. These sequences are 25.1%, 25.8%, and 23.3% identical, respectively, to Lyb-2.1 over the regions of comparison shown in Fig. 5 (179 residues for CD23, 151 residues for rat ASGPR R1, and 163 residues for human ASGPR H2). The similarities of these proteins to Lyb-2.1 are even greater if one looks at more restricted regions (e.g., 29.1% for CD23 residues 65–176, 28.3% for rat ASGPR R1 residues 60–181, and 27.5% for human ASGPR H2 residues 132–256). Fourteen residues, including four cysteines, are conserved among all four of these sequences between amino acids 143 and 267 of Lyb-2.1. These four cysteines are all within the cysteine-rich portion of Lyb-2.1. Although most of the identical residues are within the extracellular regions of these proteins, the homology between Lyb-2.1 and CD23 includes the transmembrane region and a few amino acids of the cytoplasmic domain.

DISCUSSION

We have isolated cDNA clones encoding the mouse B-cell differentiation antigen Lyb-2.1 based on the ability of these clones to yield cell surface expression of a molecule recognized by two anti-Lyb-2.1 mAbs upon transfection into COS-7 cells. Further proof that these clones encode Lyb-2.1 comes from two independent types of data. (*i*) The isolation procedure did not include any selection for B-cell specificity, yet the insert of cDNA clone pcDVLyb2.1-7 hybridized to mRNA expressed by pre-B cells and B cells, but not by antibody-secreting cells or non-B-lineage cells. This pattern of expression coincides precisely with the described pattern of Lyb-2 protein expression. (*ii*) The core protein immunoprecipitated from surface-labeled COS7 cells transfected with clone pcDVLyb2.1-7 comigrates with that immunoprecipitated from the pre-B-cell line L1.2 using the same anti-Lyb-2.1 mAb.

The evidence presented here demonstrates that Lyb-2 has an unusual orientation with respect to the membrane in that its carboxyl-terminal domain is external to the cell, and, by inference, its amino-terminal domain is within the cytoplasm. This conclusion is supported by three findings. (*i*) The amino terminus of the predicted protein sequence lacks a typical signal peptide that is characteristic of most transmembrane proteins. (*ii*) The predicted sequence contains only a single

hydrophobic segment and this segment is closer to the amino terminus than to the carboxyl terminus of the protein. Since Lyb-2 is a cell surface glycoprotein and we could not demonstrate a glycolipid linkage to the membrane, we conclude that this hydrophobic segment is the transmembrane region. These properties of Lyb-2 are characteristic of a group of proteins, including CD23 (31–33), ASGPR (34–37), transferrin receptor (38), the invariant chain associated with class II major histocompatibility complex proteins (39–41), intestinal isomaltase (42), protein band 3 of the erythrocyte plasma membrane (43), influenza virus neuraminidase (44, 45), and membrane glycoprotein PE_2 of Sindbis virus (46), all of which are believed to be oriented with their carboxyl terminus external to the cell and their amino terminus within the cell. The internal transmembrane regions of influenza virus neuraminidase, Sindbis virus glycoprotein PE_2, and the transferrin receptor have all been shown to function as signal sequences (45–47). Similarly, we predict that the transmembrane sequence of Lyb-2 serves as an internal noncleaved signal peptide. (*iii*) The most important piece of evidence for an inverted orientation of Lyb-2 is the finding that Lyb-2.1 contains N-linked glycosylmoieties, yet the only site for addition of N-linked glycosylmoieties (amino acid 136) is on the carboxyl-terminal side of the transmembrane region.

Homology searches indicate that Lyb-2.1 is homologous to two other inverted proteins—i.e., CD23 and ASGPR. CD23 and ASGPR are homologous to one another, with the greatest amount of sequence identity in the carboxyl-terminal portions of these proteins (31–33). Interestingly, this region of conservation only partially coincides (at its amino-terminal portion) with the regions of these molecules that are most similar to Lyb-2.1. These overlapping interrelationships, coupled with the finding of a series of residues conserved among all of these proteins, provide strong evidence for an evolutionary relationship of the genes encoding these molecules. We, therefore, conclude that Lyb-2, CD23, and ASGPR represent three classes of members of a gene superfamily of cell surface proteins/receptors with inverted orientation.

ASGPRs, or hepatic lectins, are cell surface receptors expressed on hepatocytes and mediate the endocytosis of glycoproteins. The rat and human molecules [of which there are two in each species, with only about 50% amino acid identity between receptors within a species (36)] recognize terminal galactose residues exposed after removal of sialic acid residues from carbohydrate groups. CD23 is also a receptor molecule. It has been identified as the B-lymphocyte (low affinity) Fc receptor for IgE (48). CD23 is expressed almost exclusively by B cells, although the same mRNA has also been detected in a human macrophage line U937 (31, 49, 50). Like Lyb-2, it is not expressed on antibody-secreting cells; however, in contrast to Lyb-2, it is absent from early B cells in bone marrow (50). Studies of the cellular biology of Lyb-2 have suggested that it too, like ASGPR and CD23, is likely to be a receptor molecule. Future studies should shed light on the possible functional similarities of the various members of this superfamily.

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