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EXTENSIVE POLYMORPHISM IN THE EXTRACELLULAR DOMAIN OF THE MOUSE B CELL DIFFERENTIATION ANTIGEN Lyb-2/CD72[1]

WILLIAM H. ROBINSON, HAN YING, M. CARRIE MICELI, AND JANE R. PARNES[2]

From the Division of Immunology and Rheumatology, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305

Lyb-2/CD72 is a 45-kDa mouse B cell surface protein that binds CD5 and has been shown to play a role in B cell proliferation and differentiation. Using the polymerase chain reaction we have isolated and sequenced cDNA clones encoding the serologically defined mouse Lyb-2^a, Lyb-2^b, and Lyb-2^c alleles. We confirmed that our full length cDNA clones encode the Lyb-2^a, -2^b, and -2^c alleles, respectively, by transfecting the isolated Lyb-2/CD72 cDNA clones into L cells and demonstrating that the transfectants bind only the appropriate allele specific anti-Lyb-2/CD72 antibodies. Sequence comparisons demonstrate that the Lyb-2/CD72 alleles are highly conserved in their cytoplasmic and transmembrane domains but exhibit a high degree of polymorphism in their extracellular domains. This polymorphism in the extracellular region involves amino acid substitutions at a minimum of 20 residues and is concentrated primarily in the membrane distal region. cDNA sequence comparisons also demonstrate two distinct seven amino acid insertion/deletions among these allelic variants. A form of Lyb-2^b cDNA lacking the sequence encoding the transmembrane region was isolated from a C57Bl/6 mouse and a CH12.LX subline. The Lyb-2/CD72 PCR products from mRNA of mice expressing Lyb-2^a and Lyb-2^c contain a DNA fragment that corresponds in size to the transmembraneless form, suggesting that these mouse strains also express this mRNA.

Lyb-2/CD72 is a 45-kDa type II integral membrane protein expressed predominantly on pre-B cells and mature B cells (1–5). Expression of the Lyb-2/CD72 protein and its mRNA is lost upon terminal differentiation of mature B cells into plasma cells (3–6). Functional studies have demonstrated that anti-Lyb-2/CD72 mAb can induce proliferation in small resting and preactivated B cells, and can synergize with IL-4 in the induction of proliferation of Ag-specific B cells (7–10). Anti-Lyb-2/CD72 mAb inhibit the antibody response by splenic B cells to T cell-dependent Ag, but not to T cell-independent Ag (11, 12). Treatment of B cells with anti-Lyb-2/CD72 mAb also induces an increase in surface expression of class II MHC proteins, mobilization of small amounts of cytoplasmic free Ca^{2+}, and an increase in the metabolism of phosphatidylinositol (13–15). Recently, CD5 (Ly-1), a cell surface glycoprotein expressed on all mature T cells and a specific subpopulation of B cells, has been identified as the natural ligand for Lyb-2/CD72 (16–19). The putative CD5-Lyb-2/CD72 interaction may play a critical role in T and B cell communication via direct cell-cell contact as well as in T and B cell activation and proliferation.

Allelic polymorphism was one of the first described properties of Lyb-2/CD72. This polymorphism was characterized using mAb specific for two distinct allelic forms of the protein (anti-Lyb-2.1 and anti-Lyb-2.3) and conventional antisera (absorbed or unabsorbed). These reagents identified five serologic specificities defining four allelic forms of mouse Lyb-2/CD72: Lyb-2^a (epitopes 1, 4, and 5), Lyb-2^b (epitopes 2 and 4), Lyb-2^c (epitope 3), and Lyb-2^d (epitope 1) (20). Two-dimensional chymotryptic peptide mapping of Lyb-2/CD72 protein isolated from different strains of inbred mice revealed three distinct patterns that correspond to the serologically defined Lyb-2^a, -2^b, and -2^c forms (20). No differences were detected by this method between Lyb-2^a and Lyb-2^d.

We have previously isolated cDNA clones encoding mouse Lyb-2^a (Lyb-2$^{a.1}$) and its human homolog (4, 5). We recently demonstrated that human Lyb-2 is the same molecule as the serologically defined human B cell protein CD72 (21). To further investigate the nature and degree of polymorphism of mouse Lyb-2/CD72 we have isolated cDNA clones encoding Lyb-2^b and Lyb-2^c as well as an alternative form of Lyb-2^a, Lyb-2$^{a.2}$. Comparison of the predicted amino acid sequences of Lyb-2^a, -2^b, and -2^c reveals polymorphism at 20 or more amino acid residues in the predicted extracellular portion of the molecule. This polymorphism can account for the observed serological differences and may provide clues as to functionally important regions in the Lyb-2/CD72 molecule.

MATERIALS AND METHODS

Mice, cell lines, and mAb. Adult mice were obtained from the following sources: AKR from Dr. I. Weissman (Stanford University), C57Bl/6 (B6) from Dr. H. O. McDevitt (Stanford University) and DBA/2, BALB/c, and C57Bl/10 (B10) from The Jackson Laboratory (Bar Harbor, ME). Hybridoma cells producing anti-Lyb-2.1 mAb 10.1.D2 were a gift of D. E. Moiser (Medical Biology Institute, La Jolla, CA). Anti-Lyb-2.2 conventional antiserum (SJL X CE vs A.SW spleen, unabsorbed) and ascites fluid containing the anti-Lyb-2.3 mAb 82–9.2 were gifts of Dr. S. Kimura (Sloan-Kettering Cancer Center, New York, NY). The CH12.LX cell line, derived from a B cell lymphoma of a B10 mouse, was the gift of Dr. G. Haughton (University of North

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[2] Address correspondence and reprint requests to Dr. Jane R. Parnes, Division of Immunology and Rheumatology, Department of Medicine, P-306, Stanford University School of Medicine, Stanford, CA 94305-5111.

Carolina, Chapel Hill, NC). A subline of CH12.LX originally obtained from Dr. G. Haughton was provided by Dr. A. Zlotnik (DNAX Research Institute, Palo Alto, CA). CH12.LX, L cells, and Cos-7 cells were grown in DMEM (GIBCO, Grand Island, NY) supplemented with 1 mM sodium pyruvate, 10 mM HEPES (Applied Scientific, San Franscisco, CA), penicillin (100 U/ml), streptomycin (100 μg/ml) (Flow Laboratories Inc., McLean, VA), and 10% heat-inactivated FCS (Gemini Bio Products Inc., Calabasas, CA).

Isolation of Lyb-2/CD72 cDNA clones. Total RNA was isolated from the CH12.LX cell line and from spleen of adult DBA/2, BALB/c, B6, B10, and AKR mice using RNAzol (Cinna/Biotecx, Friendswood, TX) according to the manufacturer's instructions. Lyb-2/CD72 cDNA was enzymatically amplified using the PCR[3] as follows (22, 23): 1 μg of total cellular or splenic RNA was reverse transcribed into cDNA in the presence of 20 ng of oligo(dT) using Moloney murine leukemia virus reverse transcriptase. PCR was performed for 30 cycles using 100 pmol of the following primers that were chosen based on sequences in the 5′ and 3′ untranslated regions and include *Eco*R1 sites for cloning purposes: AKR Lyb-2^c cDNA were amplified using the 5′ primer: 5′-TACGAATTCAGCTGGAAGACTGTGAAGCAG-3′ and the 3′ primer: 5′-ACTGAATTCCATCCATCCACTCAGCGGAGC-3′. The Lyb-2/CD72 cDNA from DBA/2 and B6 mice were amplified using the 5′ primer: 5′-TACGAATTCAGGTGTGGGCCAGTGGATTAGCT-3′ and the 3′ primer: 5′-CTAGAATTCTGC-CTGGCTGGCTCCGGGCCAT-3′. The transmembraneless cDNA clones from the CH12.LX subline were isolated using the 5′ primer: 5′-TACGAATTCAGCTGGAAGACTGTGAAGCAG-3′ and the 3′ primer: 5′-CTAGAATTCTGCCTGGCTGGCTCCGGGCCAT-3′. Amplified Lyb-2/CD72 cDNA was then cloned into the Bluescript SK^+ vector at the *Eco*R1 site.

Nucleotide sequence analysis. Two independent clones from DBA/2, two from B6, two from the CH12.LX subline expressing the transmembraneless form, and six from AKR PCR amplified Lyb-2/CD72 cDNA were subcloned into Bluescript SK^+ and sequenced fully in both directions using the dideoxy chain termination method (24) with synthetic oligonucleotide primers. One of the AKR Lyb-2^c clones had two bp changes as compared to the other five sequenced clones: a T instead of an A at the second bp of the GAG insertion between positions 950 and 951 of the Lyb-$2^{a.2}$ sequence, and an A instead of a G at position 953 (Fig. 1). No other bp changes were seen in any other clones from a single mouse strain or cell line.

Immunofluorescence analysis. L cells were transfected with the pBJ1-Neo expression vector (25) with the Lyb-2/CD72 cDNA inserted at the *Eco*R1 site using Lipofectin Reagent (BRL, Gaithersburg, MD) according to the manufacturer's instructions. Transfectants were selected by growth in the presence of 1.2 mg/ml G418 (GIBCO). In addition, Cos-7 cells were transiently transfected with the pCDV3 expression vector (26) with the Lyb-2/CD72 cDNA inserted at the *Eco*R1 site using the DEAE-dextran method (27). Transfected or untransfected cells were stained with 2 μg of ascites fluid or 1 μl conventional antiserum per 10^6 cells, followed by Texas red conjugated goat anti-mouse IgG and IgM-specific antibodies (TAGO, Burlingame, CA). Immunofluorescence was determined by analysis on a modified FACS II (Becton Dickinson, Mountain View, CA).

RESULTS

Isolation and sequence of cDNA clones encoding mouse Lyb-$2^{a.2}$, Lyb-2^b, and Lyb-2^c. PCR was used to amplify Lyb-2/CD72 cDNA encoding allelic variants of Lyb-2/CD72 from reverse transcriptase reactions on total RNA from spleen of several mouse strains. PCR primers were designed based on sequences in the 5′ and 3′ untranslated regions of the original Lyb-$2^{a.1}$ clone (from C57L) (4) and *Eco*R1 sites were included in the flanking region of the primers to facilitate subcloning. The PCR products were subcloned into the Bluescript vector at the *Eco*R1 site. Full length Lyb-$2^{a.2}$, Lyb-2^b, and Lyb-2^c cDNA were isolated from total splenic RNA from DBA/2, B6, and AKR mice, respectively. The cDNA each contain a single long open reading frame beginning at the ATG at nucleotide 33 (of the Lyb-$2^{a.2}$ clone). Lyb-$2^{a.2}$ has an open reading frame of 361 amino acids, Lyb-2^b of 354 amino acids, and Lyb-2^c of 348 amino acids (Fig. 1). The Lyb-$2^{a.2}$ sequence differs from that of the original Lyb-$2^{a.1}$ clone (open reading frame of 354 amino acids) (4) only by a 21 bp insertion of nucleotides 870 to 890. This insertion is lacking in our Lyb-2^b and Lyb-2^c clones (Fig. 1). Based on the sequence of genomic Lyb-2^a clones, this Lyb-$2^{a.2}$ insertion can be attributed to alternative splicing of the initial mRNA transcript due to the presence of two functional 3′ splice sites (H. Ying and J. R. Parnes, manuscript in preparation). We are currently investigating whether B cells of a given mouse strain can only use one or both of these 3′ splice sites. Lyb-2^c as compared to both Lyb-2^a and Lyb-2^b has a 3-bp insertion of a GAG between positions 950 and 951, and a 21-bp deletion from position 966 to 986 (Fig. 1). The latter deletion also appears to result from usage of an alternative 3′ splice site, and again, we cannot yet say with certainty whether a given mouse strain splices in only one of the two ways.

Comparison of mouse Lyb-2^a, Lyb-2^b, Lyb-2^c, and human Lyb-2/CD72 protein sequences. The predicted amino acid sequences of mouse Lyb-$2^{a.2}$, Lyb-2^b, and Lyb-2^c reveal a surprisingly high degree of polymorphism among the three allelic forms. Lyb-$2^{a.2}$ differs from Lyb-2^b and Lyb-2^c by a total of 23 and 26 amino acid substitutions, respectively, and Lyb-2^b differs from Lyb-2^c by a total of 24 amino acid substitutions (Fig. 2; Table I). Almost all of these amino acid substitutions are in the extracellular domain (i.e., carboxyl-terminal to the transmembrane region), and the greatest variation in sequence is in the membrane distal region (residues 271 to 361) (Fig. 2; Table I). Comparisons of the cytoplasmic, transmembrane, membrane proximal region (residues 117 to 270) of the extracellular, and membrane distal region (residues 271 to 361) of the extracellular domains reveal 98, 95, 92, and 75% identical residues conserved among the three different mouse alleles (ignoring gaps in alignment). Notably it is the membrane distal region of the extracellular domain that also contains the three insertion/deletions that we have found: a seven amino acid insertion of amino acids 280 to 286 in Lyb-$2^{a.2}$ as compared to Lyb-$2^{a.1}$, -2^b, and -2^c, a single amino acid insertion between residues 306 and 307 in Lyb-2^c as compared to all other forms, and a seven amino acid deletion from amino acids 312 to 318 in Lyb-2^c as compared to the other strains (all amino acid positions refer to the numbering for Lyb-$2^{a.2}$ in Fig. 2). Thus, the membrane distal region of the extracellular domain is far less conserved than the other regions of the Lyb-2/CD72 molecule. We have previously shown that the lack of conservation of this region also extends to comparisons between mouse and human Lyb-2/CD72 (5). Comparisons of the cytoplasmic, transmembrane, membrane proximal extracellular (residues 117 to 270), and membrane distal extracellular (residues 271 to 361) regions show 63, 76, 57, and 31% identical residues, respectively between mouse Lyb-$2^{a.2}$ and human Lyb-2/CD72. Of the 16 predicted cysteines in the Lyb-2^a protein, 15 are conserved among the three mouse alleles and, 13 of these are also present in the human protein. The single N-linked glycosylation site is conserved among the mouse alleles and in human Lyb-2/CD72. Lyb-2/CD72 contains a region resembling a leucine zipper (28), with leucine occurring every 7th residue between residues 143 and 171. This pattern of repetitive leucines is conserved within all the mouse alleles and between mouse and human Lyb-2/CD72. However, this region differs from leucine zipper domains

[3] Abbreviation used in this paper: PCR, polymerase chain reaction.

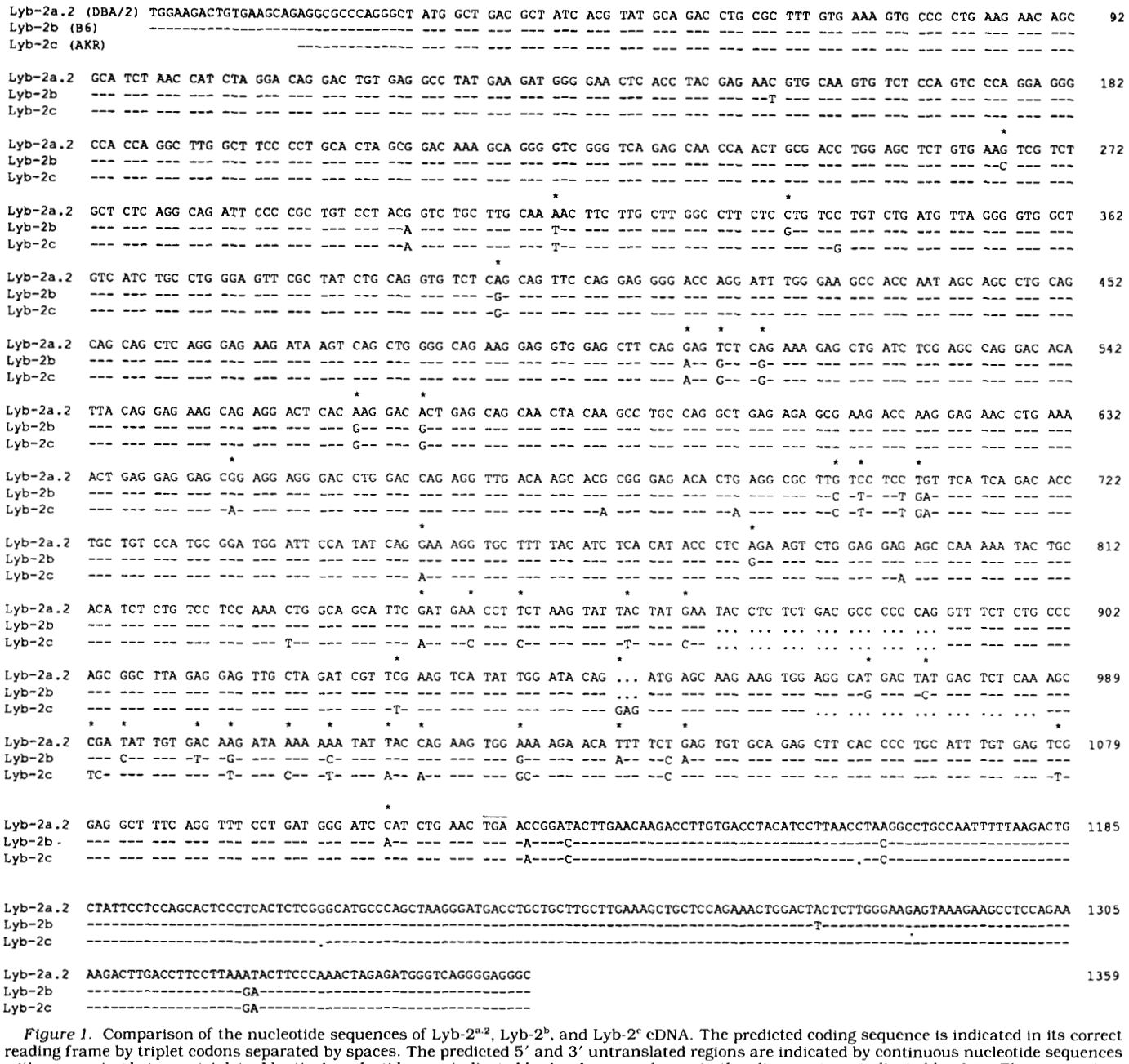

Figure 1. Comparison of the nucleotide sequences of Lyb-$2^{a.2}$, Lyb-2^{b}, and Lyb-2^{c} cDNA. The predicted coding sequence is indicated in its correct reading frame by triplet codons separated by spaces. The predicted 5′ and 3′ untranslated regions are indicated by continuous nucleotide sequences with no spacing between triplets. Identical nucleotides are indicated by *hyphens*, and gaps in the alignment are indicated by *dots*. The termination codon is overscored. The number of the last nucleotide in each line is indicated in the right margin. Nucleotide changes conferring variation in the amino acid sequence are identified by an asterisk above the first polymorphic nucleotide in the relevant triplet codon. The Lyb-$2^{a.1}$ sequence reported by Nakayama et al. (4) differs from the Lyb-$2^{a.2}$ sequence reported here only in that the 21-bp insertion from nucleotide 870–890 present in Lyb-$2^{a.2}$ is absent in Lyb-$2^{a.1}$.

in transcription factors in that it lacks an adjacent basic region that could bind DNA. This leucine zipper-like motif may be involved in homo- or heterodimerization of Lyb-2/CD72.

Isolation of Lyb-2^{b} cDNA lacking a transmembrane region. In addition to full-length clones, Lyb-2^{b} clones missing 162 bp from nucleotide 223 to 384 (relative to Lyb-$2^{a.2}$) were isolated from total splenic RNA from a B6 mouse, and also as the only form of Lyb-2^{b} cDNA from one subline of CH12.LX (provided by A. Zlotnik, DNAX Research Institute, Palo Alto, CA) (Fig. 3). Based on the genomic sequence (H. Ying and J. R. Parnes, manuscript in preparation), we believe these variant cDNA result from the alternative splicing of the Lyb-2/CD72 mRNA to exclude exons 3 and 4. The absent sequence corresponds precisely to the product that would result from the splicing out of exons 3 and 4. Exons 3 and 4 encode 32 amino acids of the cytoplasmic tail, all 21 amino acids of the predicted transmembrane region, and 2 amino acids of the extracellular domain. Southern blot analysis using genomic DNA and isolated genomic clones have demonstrated only a single copy of the Lyb-2/CD72 gene in the mouse and human genomes (5) (E. Nakayama and J. R. Parnes, unpublished observations). Thus, it is likely that alternative mRNA splicing accounts for the production of this variant transcript. The exclusion of these 162 bp encoded by exons 3 and 4 does not alter the reading frame of the extracellular domain or the normal termination codon. If translated, the variant Lyb-2^{b} mRNA represented by these clones would result in a shortened

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Lyb-2a.2  MADAITYADLRFVKVPLKNSASNHLGQDCEAYEDGELTYENVQVSPVPGGPPGLASPALADKAGVGSEQPTATWS   75
Lyb-2b    ----------------------------------------------------------------------------
Lyb-2c    ----------------------------------------------------------------------------
Lyb-2a.1  ----------------------------------------------------------------------------
human     --E----------A---K-I-SR----PG-DD---I-------PA-L-V-SS---SV-G---A-K------S-R

                                  ____________________                    •     +     +
Lyb-2a.2  SVKSSALRQIPRCPTVCLQNFLLGLLLSCLMLGVAVICLGVRYLQVSQQFQEGTRIWEATNSSLQQQLREKISQL  150
Lyb-2b    --N----------------Y------V--------------------R--------------------------
Lyb-2c    --------------------Y-----------------------R--------------------------
Lyb-2a.1  ----------------------------------------------------------------------------
human     A-T-P-VGR-LP-R-T--RYL------T--L---TA-----------L-QTN-VL-V-----R----L--T--

                +     +     +
Lyb-2a.2  GQKEVELQESQKELISSQDTLQEKQRTHKDTEQQLQACQAERAKTKENLKTEEERRRDLDQRLTSTRETLRRLSS  225
Lyb-2b    --------KAR----------------E-A-------------------------------------FF-
Lyb-2c    --------KAR----------------E-A-------------------Q-----------------FF-
Lyb-2a.1  ----------------------------------------------------------------------------
human     --SAED--G-RR--AQ--EA--VE--A-QAA-G-------D-Q----T-QS--QQ--A-E-K-SNMENR-KPFFT

Lyb-2a.2  CSS.DTCCPCGWIPYQERCFYISHTLRSLEESQKYCTSLSSKLAAFDEPSKYYYEYLSDAPQVSLPSGLEELLDR  299
Lyb-2b    D--.----------------------G-------------------------.......-------------
Lyb-2c    D--.------------K---------------------------ND-P--F-Q.......-------------
Lyb-2a.1  ---.----------------------------------------.......-------------
human     -G-A-----S---MH-KS-----L-SKNWQ----Q-ET------T-S-IYPQSHS-YFLN..SL--N-GS.....

Lyb-2a.2  SKSYWIQ.MSKKW........RHDYDSQSRYCDKIKKYYQKWKRTFSECAELHPCICESEAFRFPDGIHLN       361
Lyb-2b    -------.-----.........-Q-S-----H-VR--T-----E--I-K----------------------N--
Lyb-2c    L------E-----..............-S---M-QI-NK--A---------------L------------
Lyb-2a.1  -------.-----.........----------------------------------------------------
human     GN---TGLS-N-DWKLTDDTQ-TRTYA--SK-N-VH-TWSW-TLESES-RSSL-Y---MT------
```

Figure 2. Comparison of the predicted amino acid sequences of mouse Lyb-$2^{a.2}$, Lyb-2^{b}, Lyb-2^{c}, Lyb-$2^{a.1}$, and human Lyb-2/CD72. Identical residues are indicated by hyphens and gaps in the alignment are indicated by *dots*. The number of the last amino acid in each line is indicated in the right margin. The site of N-linked glycosylation is indicated by a *solid circle*. The predicted transmembrane region is overscored.

TABLE I

Percentage identical amino acids in comparing protein domains of the Lyb-2/CD72 alleles[a]

Lyb-2 Allele	Cytoplasmic		Transmembrane		Prox. Extracell.		Dist. Extracell.	
	Subst./tot. res.,	% ident.	Subst./tot. res.,	% ident.	Subst./tot. res.,	% ident.	Subst./tot. res.,	% ident.
Lyb-2^{a} vs -2^{b}	2/95	98	1/21	95	10/154	94	10/84	88
Lyb-2^{a} vs -2^{c}	1/95	99	0/21	100	11/154	93	14/77	82
Lyb-2^{b} vs -2^{c}	1/95	99	1/21	95	3/154	98	19/77	75
Lyb-2^{a} vs human	34/93	63	5/21	76	65/153	57	51/74	31

[a] Domains compared are the cytoplasmic, transmembrane, membrane proximal extracellular (prox. extracell.) (residues 117 to 270), and membrane distal extracellular (dist. extracell.) (residues 271 to 361). The total number of residues (tot. res.) in each domain (excluding all gaps in the alignment) along with the number of substitutions (subst.) within that domain are indicated for each comparison. The percent identical residues (% ident.) was calculated by the equation (total residues − substitutions)/total residues and expressed as a percent.

```
63                                                                              90
Gly Val Gly Ser Glu Gln Pro Thr Ala Thr Trp Ser Ser Val Asn Ser Ser Ala Leu Arg Gln Ile Pro Arg Cys Pro Thr Val
 GG GTC GGG TCA GAG CAA CCA ACT GCG ACC TGG AGC TCT GTG AAG TCG TCT GCT CTC AGG CAG ATT CCC CGC. TGT CCT ACG GTC

          91         <-CY ____________________________________________________________________________ 117
          Cys Leu Gln Tyr Phe Leu Leu Gly Leu Leu Val Ser Cys Leu Met Leu Gly Val Ala Val Ile Cys Leu Gly Val Arg Tyr
          TGC TTG CAA AAC TTC TTG CTT GGC CTT CTC CTG TCC TGT CTG ATG TTA GGG GTG GCT GTC ATC TGC CTG GGA GTT CGC T

TTG GCT TCC CCT GCA CTA GCG GAC AAA GCA GAT CTG CAG GTG TCT CAG CAG TTC CAG GAG GGG ACC AGG ATT
Leu Ala Ser Pro Ala Leu Ala Asp Lys Ala Asp Leu Gln Val Ser Arg Gln Phe Gln Glu Gly Thr Arg Ile
53  54  55  56  57  58  59  60  61  62      118 119 120 121 122 123 124 125 126 127 128 129 130
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Figure 3. Sequence of an Lyb-2^{b} cDNA encoding a transmembrane-minus polypeptide chain. Nucleotide and translated amino acid sequences from the 5′ coding region of the transmembraneless cDNA clones isolated from a B6 mouse and a CH12.LX subline (provided by A. Zlotnik, DNAX) are shown at the *bottom*. The site of the 162-bp deletion in these clones is indicated, and the nucleotide and translated amino acid sequence of the deleted region (derived from full length Lyb-2^{b} clones isolated from a B6 mouse and the CH12.LX subline obtained directly from G. Haughton, University of North Carolina) is shown bracketed at the *top*. Numbers refer to amino acid positions in the full length clone. The codons encoding the amino acids at position 63 (GGG, glycine) and 117 (TAT, tyrosine) in the mRNA represented by the full length B6 Lyb-2/CD72 clones are shown spliced together between their first and second bases to form a new codon (GAT) that generates an aspartic acid residue in the translated protein represented by the transmembraneless Lyb-2^{b} clones. The predicted transmembrane region of the full-length clone is overscored and CY indicates the cytoplasmic tail.

protein of 300 amino acids that lacks a transmembrane region and 32 amino acids of its cytoplasmic tail proximal to the membrane. Immunofluorescence staining showed no cell surface expression of Lyb-2/CD72 on the CH12.LX cell line from which the transmembraneless form of cDNA was isolated or on Cos-7 cells transfected with the transmembraneless Lyb-2^b cDNA inserted into the pCDV3 expression vector (data not shown). In contrast, another CH12.LX subline (obtained directly from G. Haughton, University of North Carolina, Chapel Hill, NC) does express surface Lyb-2/CD72, as do Cos-7 cells transfected with the full length Lyb-2^b cDNA inserted into pCDV3 (data not shown). When electrophoresed on an agarose gel the PCR products from Lyb-$2^{a.2}$ mice (DBA/2), Lyb-2^b mice (BALB/c, B6, and B10), and Lyb-2^c mice (AKR) showed two bands differing by about 0.16 kb, corresponding in size to both the full-length message including the sequences from exons 3 and 4 and the short message that is missing these sequences (data not shown). We are currently investigating the transmembraneless mRNA to determine whether it is expressed as a protein, and if so, what its function might be. In addition, a form of Lyb-2^b mRNA missing exon 3 sequence (which encodes part of the cytoplasmic tail) but including exon 4 sequence (which encodes the transmembrane region) was isolated from a B6 mouse.

Cell surface staining of L cell transfectants with anti-Lyb-2.1 mAb, anti-Lyb-2.2 conventional antiserum, and anti-Lyb-2.3 mAb. We confirmed that the Lyb-2/CD72 cDNA clones isolated from DBA/2, B6, and AKR mice did in fact encode the serologically defined Lyb-2^a, -2^b, and -2^c alleles by transfecting the cDNA inserted into the pBJ1-Neo expression vector into L cells. These transfectants were stained with Lyb-2/CD72-specific mAb and antiserum, and analyzed using the FACS (Fig. 4). The pBJ1-Neo-DBA/2 cDNA transfectants are recognized strongly with the anti-Lyb-2.1 mAb 10.1.D2 and at a low level by anti-Lyb-2.2 antiserum, but were not stained by the anti-Lyb-2.3 mAb 82–9.2 (Fig. 4, *A-C*). The anti-Lyb-2.2 conventional antiserum ((SJL/J X CE)F_1 vs A.SW spleen, unabsorbed) was generated by immunizing an Lyb-2^c mouse (SJL/J and CE mice both express Lyb-2^c) with spleen cells expressing Lyb-2^b (A.SW) and was not absorbed using Lyb-2^a expressing cells (3). The fact that pBJ1-Neo-DBA/2 cDNA L cell transfectants stained weakly positive with the anti-Lyb-2.2 antiserum could be explained by the existence of common antigenic determinants on the Lyb-2^a and Lyb-2^b molecules that are not present on Lyb-2^c. The finding that pBJ1-Neo-B6 cDNA L cell transfectants stained more strongly positive with this antiserum than the pBJ1-Neo-DBA/2 transfectants supports this hypothesis, because the anti-Lyb-2.2 antiserum should contain antibodies directed against unique Lyb-2^b antigenic determinants in addition to antibodies directed against common Lyb-2^a and Lyb-2^b determinants. Furthermore, these data are consistent with previously reported antibody plus complement lysis assay results showing that the described unabsorbed anti-Lyb-2.2 antiserum reacts with spleen cells from mouse strains expressing both Lyb-2^a and -2^b, although anti-Lyb-2.2 antiserum absorbed with Lyb-2^a-expressing cells only reacts with spleen cells from mice expressing Lyb-2^b (3, 20). The pBJ-Neo-B6 cDNA transfectants stained positively with the anti-Lyb-2.2 antiserum but not with either the anti-Lyb-2.1 or -2.3 mAb (Fig. 4, *B*, *E*, and *H*). The pBJ-Neo-AKR cDNA transfectants stained positively with the anti-Lyb-2.3 mAb but not with either the anti-Lyb-2.2 antiserum or anti-Lyb-2.1 mAb (Fig. 4, *C*, *F*, and *I*). These results are consistent with the published antibody plus complement assay data for normal mouse B cells expressing the different Lyb-2/CD72 alleles (3, 20).

DISCUSSION

We have isolated cDNA clones from total splenic RNA of DBA/2, B6, and AKR mice using PCR with primers designed from the original Lyb-2^a clone (Lyb-$2^{a.1}$). The predicted protein sequences of these clones are all more than 90% identical to the original Lyb-$2^{a.1}$ sequence, strongly suggesting that they also encode Lyb-2/CD72. This conclusion is confirmed by immunofluorescence staining of transfectants expressing the Lyb-2/CD72 cDNA. L cells transfected with a DBA/2 cDNA are recognized strongly by the anti-Lyb-2.1 mAb 10.1.D2, at a low level by the unabsorbed anti-Lyb-2.2 antiserum, and not at all by the anti-Lyb-2.3 mAb 82–9.2, indicating that this cDNA encodes an Lyb-2^a protein (Lyb-$2^{a.2}$). L cells transfected with a full length B6-derived cDNA are recognized by the anti-Lyb-2.2 antiserum but not by either the anti-Lyb-2.1- or -2.3-specific mAb, indicating that this cDNA encodes Lyb-2^b. Finally, L cells transfected with an AKR cDNA are recognized by anti-Lyb-2.3 mAb but not by the anti-Lyb-2.2 antiserum or anti-Lyb-2.1-mAb, indicating that this cDNA encodes Lyb-2^c.

Protein sequence comparisons of mouse Lyb-2^a, -2^b, and -2^c alleles and human Lyb-2/CD72 show that the cytoplasmic, transmembrane, and membrane proximal region (residues 117 to 270) of the extracellular domains are highly conserved, with approximately 95% sequence identity between mouse alleles and 60% identity between human and mouse. In contrast, the membrane distal region (residues 271 to 361) of the extracellular region is highly polymorphic, with amino acid substitutions at 10 to 19 residues in comparisons among the mouse strains, resulting in 75% identical residues conserved among the three mouse alleles sequenced, and 31% identical residues in comparing human Lyb-2/CD72 to mouse Lyb-$2^{a.2}$. Notably, this region is also the site of all of the insertions/deletions that we have noted among mouse Lyb-2/CD72 alleles and of the majority of such sequence gaps in mouse vs human Lyb-2/CD72. The highly conserved regions of Lyb-2/CD72 may be functionally important. Both human and mouse CD5 bind to all three allelic forms of mouse Lyb-2/CD72 that we have examined (19). Inasmuch as CD5 binds to the external region of Lyb-2/CD72, the CD5 binding domain is most likely located in the membrane proximal region of the extracellular domain of Lyb-2/CD72, because this region is significantly more highly conserved than the membrane distal region. The high degree of amino acid sequence conservation in the cytoplasmic tail of different Lyb-2/CD72 molecules suggests that this domain may be involved in signal transduction, possibly through interaction with other proteins. It remains unclear what role the highly polymorphic distal extracellular region may play in Lyb-2/CD72 function.

Short Lyb-2^b cDNA clones missing the region hypothesized to be the transmembrane region and part of the cytoplasmic tail were isolated from B6 mice and from one

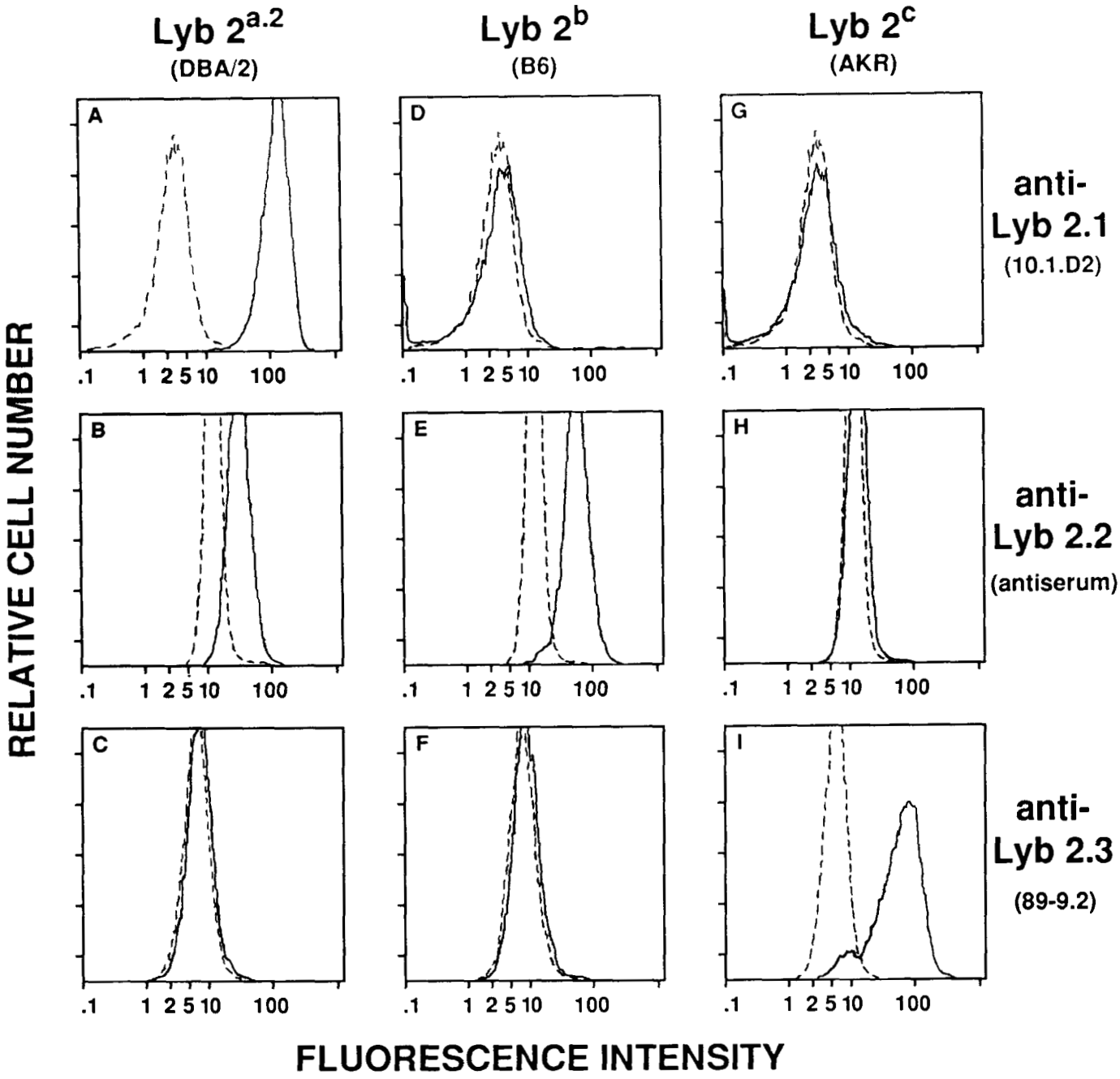

Figure 4. Cell surface staining for Lyb-2/CD72 alleles of L cells transfected with the pBJ1-Neo expression vector containing the Lyb-$2^{a.2}$, 2^b, and 2^c cDNA and analyzed using a FACS. *Vertical columns* of plots represent Cos-7 cells transfected with the Lyb-$2^{a.2}$ cDNA isolated from DBA/2 splenic RNA (*A-C*), Lyb-2^b cDNA isolated from B6 splenic RNA (*D-F*), and Lyb-2^c cDNA isolated from AKR splenic RNA (*G-I*). *Dashed lines* represent the negative controls for each column, which are the appropriate transfectants stained with Texas red-conjugated goat anti-mouse IgG- and IgM-specific secondary Ab alone. *Solid lines* represent staining of the transfectants with anti-Lyb-2.1 mAb 10.1.D2 (*A*, *D*, and *G*), anti-Lyb-2.2 conventional antiserum (*B*, *E*, and *H*), or anti-Lyb-2.3 mAb 82–9.2 (*C*, *F*, and *I*) followed by goat anti-mouse secondary antibodies.

subline of the CH12.LX cell line. The electrophoresed Lyb-2/CD72 PCR products from Lyb-2^a, Lyb-2^b, and Lyb-2^c mice showed bands that correspond to both the transmembraneless and full length forms of mRNA. Based on the genomic sequence (H. Ying and J. R. Parnes, unpublished observations) we propose that these transmembraneless mRNA arose from the splicing out of sequences encoded in exons 3 and 4. Lyb-2/CD72 is a type II inverted membrane protein that lacks an amino terminal signal peptide and contains a single long hydrophobic stretch thought to be the transmembrane domain and to serve as a noncleaved signal peptide. As a result, we believe that the transmembraneless form, if translated, will result in cytoplasmic Lyb-2/CD72 that could be degraded intracellularly or be involved in intracellular processes. We are currently investigating whether a protein product can be detected from this mRNA in mice and in the CH12.LX subline that produces only the transmembraneless mRNA.

We also isolated from B6 mice a short Lyb-2^b cDNA clone missing the region corresponding to exon 3 (hypothesized to encode part of the cytoplasmic tail) but including exon 4 (hypothesized to encode the transmembrane region). When transfected into L cells this exon 3$^-$

Lyb-2^b cDNA generates a protein product that can be detected on the cell surface using immunofluorescence staining, although no Lyb-2/CD72 surface expression is detected in L cells transfected with the exon 3^-4^- Lyb-2^b cDNA (W. H. Robinson and J. R. Parnes, unpublished observations). In addition to isolating short Lyb-2/CD72 cDNA representing differential mRNA splicing of exons 3 and 4, we isolated cDNA representing alternative splicing of the initial mRNA transcript at two different exon-intron junctions due to the presence of more than one functional splice site based on the genomic sequence (H. Ying and J. R. Parnes, manuscript in preparation). The cDNA nucleotide sequence differences that represent use of alternative splice sites include a 21-bp insertion of nucleotides 870 to 890 in Lyb-$2^{a.2}$ as compared to Lyb-$2^{a.1}$, -2^b, and -2^c as well as a second 21 bp deletion between nucleotides 966 and 986 in Lyb-2^c as compared to Lyb-$2^{a.1}$, -$2^{a.2}$, and -2^b (Fig. 1). We are currently investigating whether B cells of a given mouse strain can use only one or both of these alternative splice sites.

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