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Biochemical Identity of the Mouse Ly-19.2 and Ly-32.2 Alloantigens with the B Cell Differentiation Antigen Lyb-2/CD72[1]

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Abstract. Lyb-2/CD72 is a 45-kDa mouse B cell surface protein that binds CD5 (Ly-1) and has been shown to induce B cell proliferation upon mAb binding. The serologically defined Ly-19.2 and Ly-32.2 lymphocyte alloantigens have mouse strain distribution patterns similar to that of the Lyb-2/CD72 alleles and map to the same region on chromosome 4 as Lyb-2/CD72. Our recent isolation of the Lyb-2^a, -2^b, and -2^c cDNA has enabled us in this report to examine the relationship between Ly-19, Ly-32, and Lyb-2/CD72. A rat T cell line transfected with a mouse Lyb-2^a cDNA is recognized by Ly-19.2-specific mAb, whereas transfectants expressing the Lyb-2^b cDNA are recognized by both Ly-19.2 and Ly-32.2-specific mAb. Cell surface iodination immunoprecipitation analysis from Lyb-2^a cDNA transfectants using Lyb-2^a- and Ly-19.2-specific mAb as well as from Lyb-2^b cDNA transfectants using Lyb-2^b-, Ly-19.2-, and Ly-32.2-specific Ab, produced immunoprecipitates containing comigrating 45-kDa polypeptides. Preclearing studies with these transfectants indicate that the immunoprecipitated proteins represent the same polypeptide chain. These results demonstrate that the mouse Ly-19.2 and Ly-32.2 alloantigens are in fact the B cell differentiation Ag Lyb-2/CD72. *Journal of Immunology,* 1993, 151: 4764.

Lyb-2/CD72 is a 45-kDa type II integral membrane glycoprotein expressed on pre-B cells and mature B cells, but not on plasma cells (1–3). Lyb-2/CD72-specific mAb stimulate proliferation in small resting and preactivated B cells and inhibit the Ab response of splenic B cells to T cell-dependent Ag but not to T cell-independent Ag (4–8). Treatment of B cells with Lyb-2/CD72-specific Ab 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 (9–11). CD5 (Ly-1), a cell surface glycoprotein expressed on all mature T cells and a subpopulation of B cells, binds Lyb-2/CD72, and this interaction may play a critical role in the regulation of T and B cell activation and proliferation (12, 13).

Ly-19 and Ly-32 are lymphocyte surface proteins defined by mAb specific for the mouse Ly-19.2 and Ly-32.2 alloantigens, respectively (14, 15). The Ly-19.1 and Ly-32.1 phenotypes are associated with strains lacking reactivity to these mAb. The mouse strain distribution of the Ly-19.2 alloantigen corresponds with the Lyb-2^a and Lyb-2^b alleles, whereas that of the Ly-32.2 alloantigen corresponds with the Lyb-2^b allele alone (14–17). Typing of Lyb-2/CD72 congenic strains combined with linkage studies have shown the Ly-19 and Ly-32 loci to be tightly linked to that of Lyb-2/CD72 on mouse chromosome 4, with no observed recombinations (14, 15, 18–20). Based on data from cytotoxicity assays using B and T cell lines as well as cells isolated from lymphoid organs, investigators previously believed that Lyb-2/CD72 was expressed only on B cells (1, 2) and thus was a distinct molecule from Ly-19 and Ly-32, which are expressed on at least some T cells as well as B cells (14, 15). One previous immunoprecipitation study using splenic B cells demonstrated that the antigenic determinants recognized by Ly-19.2, Ly-32.2, and Lyb-2/

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[3] Abbreviation used in this paper: Ab, antibody.

CD72-specific mAb exist on the same molecular complex composed of 45- and 95-kDa polypeptides (20). However, these investigators could not determine which of the polypeptide chains they described correlated with epitopes recognized by the Ly-19.2-, Ly-32.2-, and Lyb-2/CD72-specific mAb. A second immunoprecipitation study, using splenic B cells and Lyb-2.1-specific mAb, proposed that the Lyb-2/CD72 complex was composed of 45- and 105-kDa polypeptides (21). Under nonreducing conditions, Ashida et al. (21) reported Lyb-2/CD72 immunoprecipitates containing higher molecular mass complexes of 160 kDa (hypothesized to represent a heterodimer of the 45-kDa Lyb-2/CD72 and 105-kDa polypeptides) and 125 kDa (hypothesized to represent a homotrimer of the 45-kDa Lyb-2/CD72 polypeptide). With our isolation of the Lyb-2^a cDNA encoding a polypeptide of predicted 45 kDa (3), the possibility arose that either the 95- or 105-kDa polypeptide could represent Ly-19 or Ly-32.

We have recently isolated cDNA clones encoding mouse Lyb-2^a, -2^b, and -2^c alleles, which are highly polymorphic in their membrane distal extracellular regions (3, 22), and their human homologue (23). We also demonstrated that human Lyb-2 is the same molecule as the serologically defined human B cell protein CD72 (24). In this report, we present immunofluorescence staining and cell surface iodination immunoprecipitation analysis using Ly-19.2-, Ly-32.2-, and Lyb-2/CD72-specific Ab on transfectants expressing the Lyb-2/CD72 cDNA to demonstrate that the serologically defined Ly-19.2 and Ly-32.2 alloagntigens are in fact the B cell differentiation Ag Lyb-2/CD72.

Materials and Methods

Cell lines and mice

NB2–6TG (NB2) is a rat T cell line obtained from H. Waldmann (University of Cambridge, Cambridge, United Kingdom). Cells were grown in RPMI 1640 (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). Transfection of NB2 cells with pBJ-neo-Lyb-$2^{a.2}$, -2^b, and -2^c cDNA expression constructs (22) was performed by electroporation using a Bio-Rad gene pulser at 675 V/cm and 960 μF according to the manufacturer's instructions. NB2-Lyb-2/CD72 cDNA transfectants were selected by growth in the presence of 1.2 mg/ml G418 (GIBCO). Lyb-$2^{a.2}$ differs from Lyb-$2^{a.1}$ by a 21-bp insertion between nucleotides 870 and 891 of the Lyb-$2^{a.2}$ cDNA sequence (22). Because both Lyb-$2^{a.1}$ and Lyb-$2^{a.2}$ are recognized by the Lyb-2.1-specific mAb 10.1.D2 and the Ly-19.2-specific mAb K10.6 (3, 22) (W. H. Robinson and J. R. Parnes, unpublished observations and data contained within this report), there is no need to distinguish between these two forms for the purposes of this paper, and we will refer to Lyb-$2^{a.2}$ as Lyb-2^a.

Ab

The Ly-19.2-specific mAb K10.6 and the Ly-32.2-specific mAb B9.689 were obtained from N. Tada (Tokai University School of Medicine, Japan). Hybridoma cells producing Lyb-2.1-specific mAb 10.1.D2 (which recognizes Lyb-$2^{a.1}$ and Lyb-$2^{a.2}$) were a gift of D. E. Moiser (Medical Biology Institute, La Jolla, CA). Lyb-2.2 conventional antiserum (SJL × CE vs A.SW spleen, unabsorbed; which recognizes Lyb-2^b), and ascites fluid containing the Lyb-2.3-specific mAb 82–9.2 (which recognizes Lyb-2^c) were gifts of Dr. S. Kimura (Sloan-Kettering Cancer Center, New York, NY).

Immunofluorescence analysis

Transfected or untransfected NB2 cells were stained with 2 μg of ascites fluid or 0.5 μg protein A-purified mAb/10^6 cells, followed by Texas red-conjugated goat anti-mouse IgG and IgM specific Ab (Tago, Burlingame, CA). Immunofluorescence was determined by analysis on a modified FACS II (Becton Dickinson) FMF.

Cell surface iodination and immunoprecipitation

NB2 cells (10^7) were surface labeled with 0.5 mCi ^{125}I (1 mCi = 37 GBq; Amersham, Arlington Heights, IL) using the lactoperoxidase method as described previously (25). Cells were washed in PBS and lysed in 0.4 ml lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.15 M NaCl, and 1 mM PMSF); 100 μl 10% protein A-Sepharose (Pharmacia, Piscataway, NJ) in lysis buffer were preloaded with 2 μg of protein G-purified 10.1.D2 or K10.6 mAb, or 10 μg of ascites fluid containing the B9.689 or 82–9.2 mAb, or 5 μl conventional antiserum. B9.689 is an IgM isotype, and goat-anti-mouse IgM-specific Ab (Jackson Immunoresearch, West Grove, PA) was used to couple B9.689 to protein A-Sepharose. NB2 cell lysates were precleared twice with 100 μl protein A-Sepharose preloaded with 5 μl normal mouse serum before undergoing sequential immunoprecipitation for 1 h at 4°C with protein A-Sepharose preloaded with the appropriate Ab. The precipitates were washed as described (26). Half of each sample was resuspended in 1× loading buffer containing 1% 2-ME (reducing), and the other half in 1× loading buffer without 2-ME (nonreducing), and the samples were electrophoresed on 10% SDS-polyacrylamide gels as described (27).

Results

Cell surface staining of NB2-Lyb-2/CD72 cDNA transfectants with Ly-19.2 and Ly-32.2-specific mAb

NB2 cells were transfected with our previously described expression vector constructs (22) encoding the Lyb-2^a, -2^b,

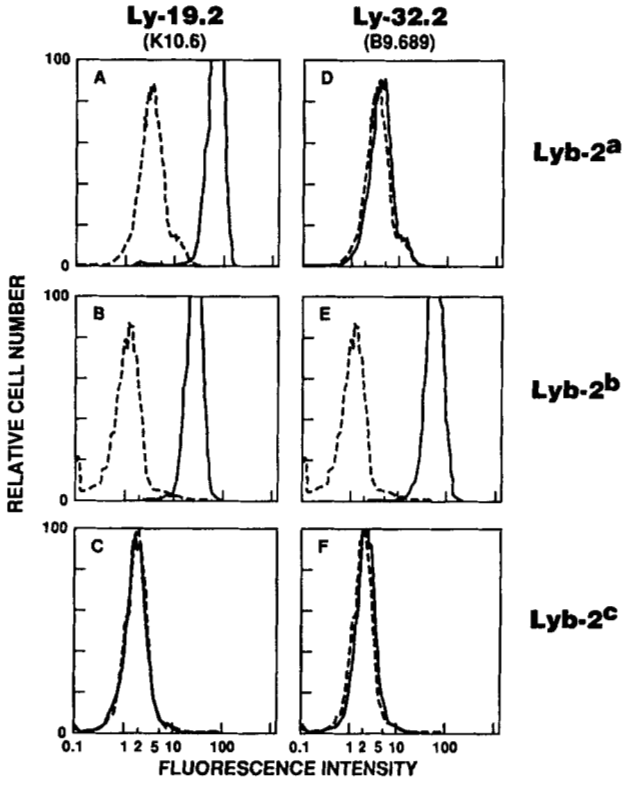

FIGURE 1. FMF analysis of NB2-Lyb-2/CD72 cDNA transfectants cell surface stained with Ly-19.2 and Ly-32.2-specific mAb. *Horizontal rows of plots* represent NB2 cells transfected with the pBJ-neo expression vector containing the Lyb-2^a cDNA (*A* and *D*), Lyb-2^b cDNA (*B* and *E*), and Lyb-2^c cDNA (*C* and *F*). *Dashed lines* represent the negative controls for each column, which are the appropriate transfectants stained with Texas red-conjugated goat anti-mouse Ig-specific secondary Ab alone. *Solid lines* represent staining of the transfectants with Ly-19.2-specific mAb K10.6 (*A, B,* and *C*) or Ly-32.2-specific mAb B9.689 (*D, E,* and *F*) plus the secondary Ab indicated above.

and -2^c alleles. The pBJ-neo-Lyb-2/CD72 NB2 transfectants were immunofluorescently stained with the Ly-19.2-specific mAb K10.6 and Ly-32.2-specific mAb B9.689 to determine the relationship between these antigenic determinants and the Lyb-2/CD72 gene product. We have previously demonstrated that L cell transfectants expressing the Lyb-2^a, -2^b, and -2^c cDNA were recognized by the appropriate Lyb-2/CD72 allele-specific Ab (22). In addition, NB2 cells stably transfected and Cos-7 cells transiently transfected with the Lyb-2/CD72 cDNA were recognized by the appropriate Lyb-2/CD72 allele-specific Ab (W. H. Robinson and J. R. Parnes, unpublished observations). As shown in Figure 1, the Ly-19.2-specific mAb K10.6 positively stained NB2 cells expressing both the Lyb-2^a and Lyb-2^b, but not the Lyb-2^c cDNA. This staining pattern is consistent with K10.6 recognizing a common antigenic determinant on the Lyb-2^a and Lyb-2^b molecules. This common antigenic determinant could be the Lyb-2.4 epitope as defined by Tung et al. (17), or a different common epitope. Based on comparison of the predicted protein sequences for the mouse Lyb-2/CD72 alleles (22), residues 271 to 279 might encode the Ly-19.2 epitope recognized by K10.6, because they are shared by the Lyb-2^a and Lyb-2^b, but not by the Lyb-2^c, polypeptides. The Ly-32.2-specific mAb B9.689 positively stained only the NB2-Lyb-2^b, and not NB2-Lyb-2^a or -2^c cDNA transfectants (Fig. 1). This staining pattern is consistent with B9.689 recognizing an antigenic determinant present only on the Lyb-2^b molecule. This Lyb-2^b-specific antigenic determinant could be the Lyb-2.2 epitope as defined by Tung et al. (17) or a different epitope unique to Lyb-2^b. Based on the comparison of the predicted protein sequences for the mouse Lyb-2/CD72 alleles, residues 321 to 338 might encode the Ly-32.2 epitope recognized by the B9.689 mAb, because they are present in the Lyb-2^b, but not in the Lyb-2^a or Lyb-2^c polypeptides. The same immunofluorescence staining results with K10.6 and B9.689 were obtained using Cos-7 cells transiently transfected or L cells stably transfected with the Lyb-2/CD72 cDNA (data not shown). These immunofluorescence staining data provide the first line of evidence that the Ly-19.2-specific mAb K10.6 recognizes a common epitope on the Lyb-2^a and Lyb-2^b molecules, whereas the Ly-32.2-specific mAb B9.689 recognizes a unique epitope present only on the Lyb-2^b molecule.

Comparison of polypeptides immunoprecipitated by Ly-19.2-, Ly-32.2-, and Lyb-2/CD72-specific Ab from transfected NB2 cells

We performed immunoprecipitations from lysates of radioiodinated transfected or untransfected NB2 cells using the Ly-19.2-specific mAb K10.6, the Ly-32.2-specific mAb B9.689, the Lyb-2.1-specific mAb 10.1.D2 (which recognizes Lyb-2^a), Lyb-2.2-specific antiserum (which recognizes Lyb-2^b), and Lyb-2.3-specific mAb 82–9.2 (which recognizes Lyb-2^c). The precipitated proteins were analyzed under reducing and nonreducing conditions (see Figs. 2–5). None of the Ab precipitated a protein of the appropriate size from untransfected NB2 cells (see Fig. 5).

When analyzed under reducing conditions, Lyb-2.1-specific mAb 10.1.D2 (which recognizes Lyb-2^a) and Ly-19.2-specific mAb K10.6 both precipitated a comigrating, indistinguishable polypeptide of 45 kDa, representing Lyb-2/CD72 from NB2-Lyb-2^a transfectants; no other prominent bands were present (Fig. 2, *Lanes 1* and *4*). When the immunoprecipitates from NB2-Lyb-2^a transfectants were analyzed under nonreducing conditions, 10.1.D2 and K10.6 immunoprecipitated indistinguishable smears of protein complexes (data not shown). Lyb-2.3-specific mAb 82–9.2 and Ly-32.2-specific mAb B9.689 did not immunoprecipitate any prominent bands from NB2-Lyb-2^a transfectants,

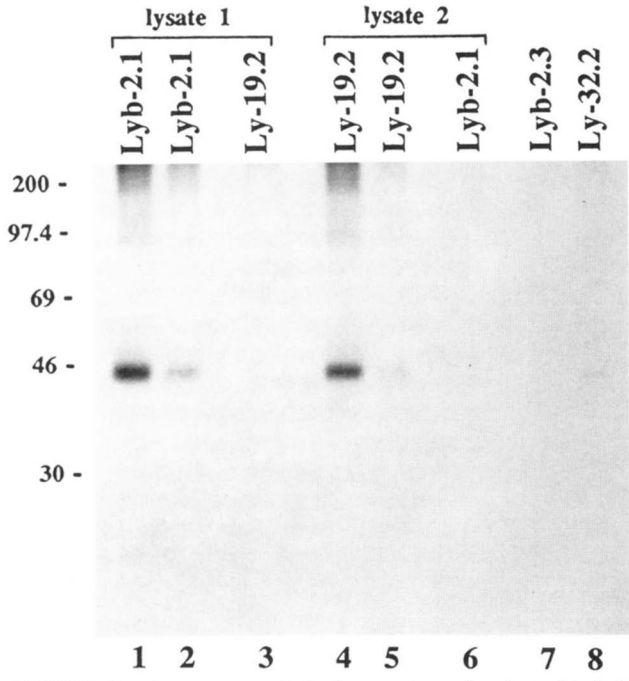

FIGURE 2. Immunoprecipitation and preclearing of Lyb-2/CD72 from cell lysates of NB2-Lyb-2^a cDNA transfectants. NB2 cells transfected with the Lyb-2^a cDNA in the pBJ-neo expression vector were surface-labeled with ^{125}I and lysed, and the lysates precleared twice with normal mouse serum before preclearing and immunoprecipitation using specific Ab. Samples were electrophoresed on a 10% SDS-polyacrylamide gel under reducing conditions. A lysate of NB2-Lyb-2^a cells was precleared twice with Lyb-2.1-specific mAb 10.1.D2 (which recognizes Lyb-2^a) (*lanes 1* and *2*) and then reprecipitated with Ly-19.2-specific mAb K10.6 (*lane 3*). A second NB2-Lyb-2^a cell lysate was precleared twice with K10.6 (*lanes 4* and *5*) and then reprecipitated with 10.1.D2 (*lane 6*). A third NB2-Lyb-2^a cell lysate was immunoprecipitated with Lyb-2.3-specific mAb 82–9.2 (which recognizes Lyb-2^c) (*lane 7*), and a fourth with Ly-32.2-specific mAb B9.689 (*lane 8*). The migration positions of comigrated ^{14}C-labeled molecular mass standards ($\times 10^{-3}$) are shown in the *left margin*.

which is consistent with their specificity for the Lyb-2^c and Ly-32.2 (Lyb-2^b) molecules, respectively (Fig. 2, *Lanes 7* and *8*).

The Lyb-2.2-specific antiserum (which recognizes Lyb-2^b), Ly-19.2-specific mAb K10.6, and Ly-32.2-specific mAb B9.689, all immunoprecipitated comigrating, indistinguishable polypeptide doublets of 45 kDa representing Lyb-2/CD72 from NB2-Lyb-2^b transfectants when analyzed under reducing conditions (Fig. 3*A*, *Lanes 2, 6,* and *10*). When the immunoprecipitates from NB2-Lyb-2^b transfectants were analyzed under nonreducing conditions, the Lyb-2.2-specific antiserum, K10.6, and B9.689 immunoprecipitated comigrating, indistinguishable 90-kDa protein complexes; no other larger protein complexes could be distinguished, although some very large complexes appeared to barely enter the gel (Fig. 3*B*, *Lanes 1, 5,* and *9*). These 90-kDa bands most likely represent homodimers of Lyb-2/CD72. Whereas the previously described 95- or 105-kDa polypeptides in reduced immunoprecipitates using the Ly-19.2-, Ly-32.2-, and Lyb-2/CD72-specific Ab could possibly represent Ly-19 or Ly-32 (14, 15), we observed no 140- to 150-kDa or larger protein complexes in our nonreduced immunoprecipitates that could represent Lyb-2/CD72 complexed to a 95- or 105-kDa polypeptide (bands representing larger protein complexes should be present even if an associated polypeptide did not surface label well with ^{125}I) (Fig. 3*B*, *Lanes 1, 5,* and *9*). Lyb-2.1-specific mAb 10.1.D2 and Lyb-2.3-specific mAb 82–9.2 did not immunoprecipitate any prominent bands from NB2-Lyb-2^b transfectants under reducing or nonreducing conditions, which is consistent with their specificity for the Lyb-2^a and Lyb-2^c molecules, respectively (reduced: Fig. 3*A*, *Lane 1*; nonreduced: Fig. 3*B*, *Lane 13*; data not shown for the 10.1.D2 immunoprecipitates).

Under reducing conditions, Lyb-2.3-specific mAb 82–9.2 (which recognizes Lyb-2^c) immunoprecipitated a comigrating, indistinguishable polypeptide doublet of 45 kDa representing Lyb-2/CD72 from NB2-Lyb-2^c transfectants when compared to the 45-kDa bands immunoprecipitated by the Ly-19.2-, Ly-32.2-, and other Lyb-2/CD72-specific Ab from the NB2-Lyb-2^a and -2^b cDNA transfectants; no other prominent bands were present (Fig. 4*A*, *Lane 2*). When analyzed under nonreducing conditions, 82–9.2 immunoprecipitated a 90-kDa protein complex, presumably representing Lyb-2/CD72 homodimers (Fig. 4*B*, *Lane 2*). Lyb-2.1-specific mAb 10.1.D2 and Ly-32.2-specific mAb B9.689 did not immunoprecipitate any prominent bands from NB2-Lyb-2^c transfectants under reducing or nonreducing conditions, which is consistent with their specificity for the Lyb-2^a and -2^b molecules, respectively (Fig. 4). Ly-19.2-specific mAb K10.6 immunoprecipitated several proteins from NB2-Lyb-2^c transfectants under reducing conditions (Fig. 4*A*, *Lane 4*), but we believe that these are nonspecific because the same protein bands are also present in K10.6, 10.1.D2, and B9.689 immunoprecipitates from untransfected NB2 cells (Fig. 5, *Lanes 1, 4,* and *5*). In addition, these nonspecific bands are present in reduced Ly-19.2 immunoprecipitates from NB2-Lyb-2^b transfectants, but are much less intense than the prominent 45-kDa doublet representing Lyb-2/CD72 (Fig. 3*A*, *Lane 6*).

These data demonstrate that under both reducing and nonreducing conditions, the polypeptides immunoprecipitated by the Ly-19.2-specific mAb K10.6 from NB2-Lyb-2^a and NB2-Lyb-2^b transfectants as well as by the Ly-32.2-specific mAb B9.689 from NB2-Lyb-2^b transfectants are identical in size to the mouse Lyb-2/CD72 polypeptide immunoprecipitated by the Lyb-2/CD72-specific mAb 10.1.D2, 82–9.2, and the Lyb-2.2-specific antiserum.

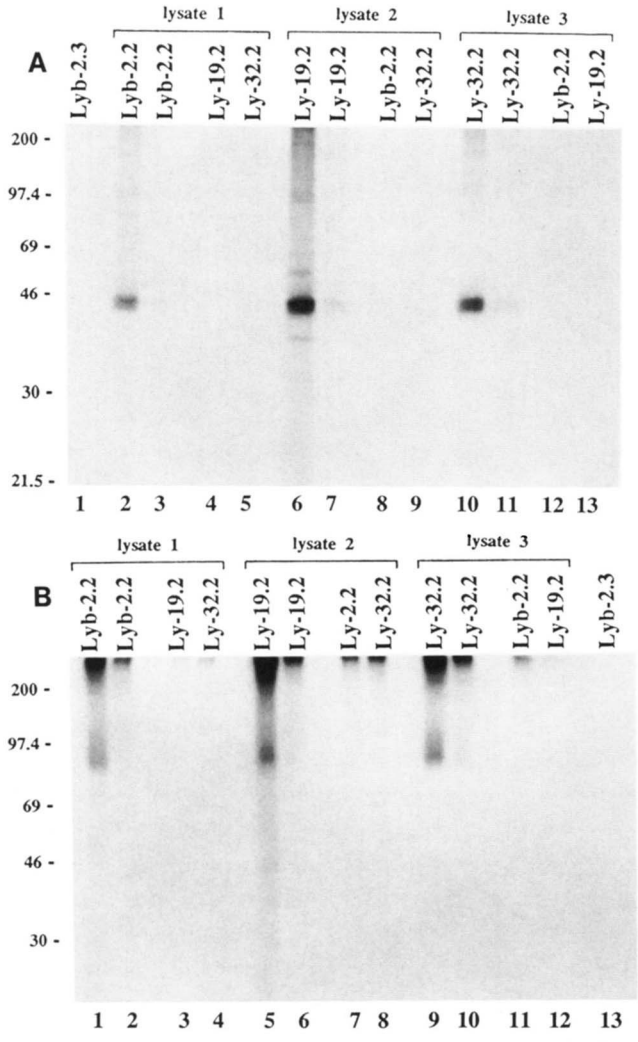

FIGURE 3. Immunoprecipitation and preclearing of Lyb-2/CD72 from cell lysates of NB2-Lyb-2^b cDNA transfectants. NB2 cells transfected with the Lyb-2^b cDNA in the pBJ-neo expression vector were surface-labeled with ^{125}I, lysed, and the lysates precleared twice with normal mouse serum before preclearing and immunoprecipitation using specific Ab. Samples were electrophoresed on a 10% SDS-polyacrylamide gel under reducing (*A*) or nonreducing (*B*) conditions. A lysate of NB2-Lyb-2^b cells was precleared twice with Lyb-2.2-specific antiserum (which recognizes Lyb-2^b) (reduced: *A, lanes 2* and *3*; nonreduced: *B, lanes 1* and *2*), following which the lysate was divided in half and reprecipitated with Ly-19.2-specific mAb K10.6 (reduced: *A, lane 4*; nonreduced: *B, lane 3*) or Ly-32.2-specific mAb B9.689 (reduced: *A, lane 5*; nonreduced: *B, lane 4*). A second NB2-Lyb-2^b cell lysate was precleared twice with K10.6 (reduced: *A, lanes 6* and *7*; nonreduced: *B, lanes 5* and *6*), following which the lysate was divided in half and reprecipitated with Lyb-2.2-specific antiserum (reduced: *A, lane 8*; nonreduced: *B, lane 7*) or B9.689 (reduced: *A, lane 9*; nonreduced: *B, lane 8*). A third NB2-Lyb-2^b cell lysate was precleared twice with B9.689 (reduced: *A, lanes 10* and *11*; nonreduced: *B, lanes 9* and *10*), following which the lysate was divided in half and reprecipitated with Lyb-2.2-specific antiserum (reduced: *A, lane 12*; nonreduced: *B, lane 11*) or K10.6 (reduced: *A, lane 13*; nonreduced: *B, lane 12*). A fourth NB2-Lyb-2^b cell lysate was immunoprecipitated with Lyb-2.3-specific mAb 82–9.2 (which recognizes Lyb-2^c) (reduced: *A, lane 1*; nonreduced: *B, lane 13*). The migration positions of comigrated ^{14}C-labeled molecular mass standards ($\times 10^{-3}$) are shown in the *left margin*.

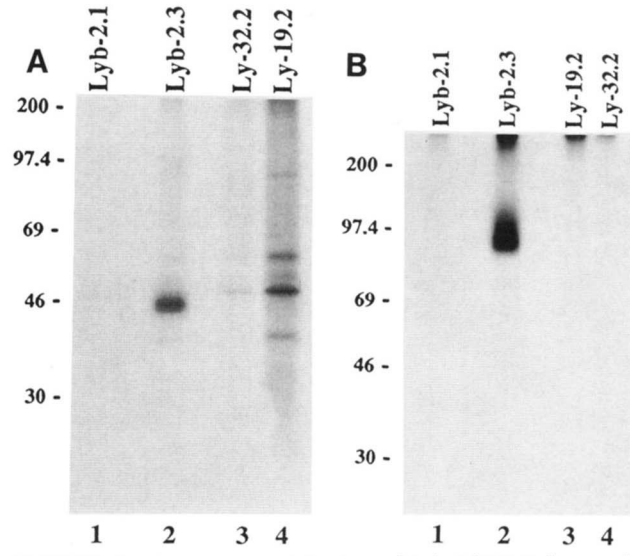

FIGURE 4. Immunoprecipitation of Lyb-2/CD72 from cell lysates of NB2-Lyb-2^c cDNA transfectants. NB2 cells transfected with the Lyb-2^c cDNA in the pBJ-neo expression vector were surface-labeled with ^{125}I and lysed, and the lysates precleared twice with normal mouse serum before preclearing and immunoprecipitation using specific Ab. Samples were electrophoresed on a 10% SDS-polyacrylamide gel under reducing (*A*) or nonreducing (*B*) conditions. Individual NB2-Lyb-2^c cell lysates were immunoprecipitated with Lyb-2.1-specific mAb 10.1.D2 (which recognizes Lyb-2^a) (reduced: *A, lane 1*; nonreduced: *B, lane 1*), Lyb-2.3-specific mAb 82–9.2 (which recognizes Lyb-2^c) (reduced: *A, lane 2*; nonreduced: *B, lane 2*), Ly-19.2-specific mAb K10.6 (reduced: *A, lane 4;* nonreduced: *B, lane 3*), and Ly-32.2-specific mAb B9.689 (reduced: *A, lane 3*; nonreduced: *B, lane 4*). The migration positions of comigrated ^{14}C-labeled molecular mass standards ($\times 10^{-3}$) are shown in the *left margin*.

Preclearing of the NB2-Lyb-2/CD72 cell immunoprecipitates with the Ly-19.2-, Ly-32.2-, and Lyb-2/CD72-specific Ab.

Although the above data represent strong evidence that Ly-19, Ly-32, and Lyb-2/CD72 are identical molecules, we performed preclearing experiments to confirm that Ly-19.2-, Ly-32.2-, and Lyb-2/CD72-specific Ab recognize the same polypeptide. A lysate of radioiodinated NB2-Lyb-2^a transfectants was precleared twice with Lyb-2.1-specific mAb 10.1.D2 (which recognizes Lyb-2^a) and then reprecipitated with Ly-19.2-specific mAb K10.6 (Fig. 2, *Lanes 1* to *3*). A second NB2-Lyb-2^a lysate was precleared twice with K10.6 and then reprecipitated with

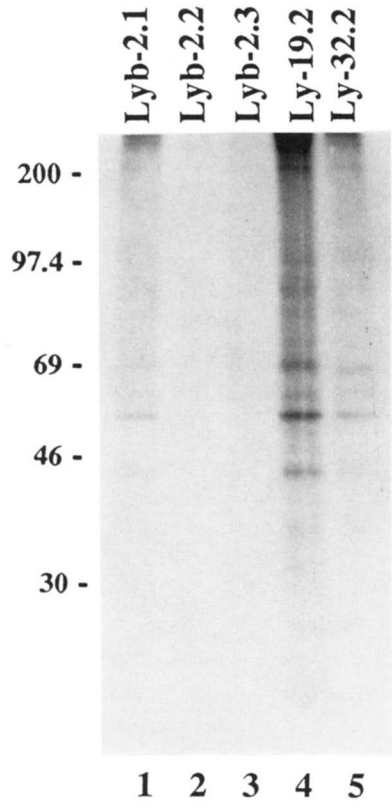

FIGURE 5. Immunoprecipitation using Lyb-2/CD72-, Ly-19.2-, and Ly-32.2-specific mAb from cell lysates of untransfected NB2 cells. Untransfected NB2 cells were surface labeled with ^{125}I and lysed, and the lysates precleared twice with normal mouse serum before preclearing and immunoprecipitation using specific Ab. Samples were electrophoresed on a 10% SDS-polyacrylamide gel under reducing conditions. Untransfected NB2 cells were immunoprecipitated with Lyb-2.1-specific mAb 10.1.D2 (which recognizes Lyb-2^a; *lane 1*), Lyb-2.2-specific antiserum (which recognizes Lyb-2^b; *lane 2*), Lyb-2.3-specific mAb 82–9.2 (which recognizes Lyb-2^c; *lane 3*), Ly-19.2-specific mAb K10.6 (*lane 4*), and Ly-32.2-specific mAb B9.689 (*lane 5*). The migration positions of comigrated ^{14}C-labeled molecular mass standards ($\times 10^{-3}$) are shown in the *left margin*.

10.1.D2 (Fig. 2, *Lanes 4* to *6*). After preclearing NB2-Lyb-2^a lysates twice with either 10.1.D2 or K10.6, no protein band could be detected upon reprecipitation with K10.6 or 10.1.D2, respectively (Fig. 2, *Lanes 3* and *6*).

A lysate of radioiodinated NB2-Lyb-2^b transfectants was precleared twice with the Lyb-2.2-specific antiserum (which recognizes Lyb-2^b) and then divided in half and reprecipitated with Ly-19.2-specific mAb K10.6 or Ly-32.2-specific mAb B9.689 (reduced: Fig. 3*A*, *Lanes 2* to *5*; nonreduced: Fig. 3*B*, *Lanes 1* to *4*). A second NB2-Lyb-2^b lysate was precleared twice with K10.6 and then divided in half and reprecipitated with Lyb-2.2-specific antiserum or B9.689 (reduced: Fig. 3*A*, *Lanes 6* to *9*; nonreduced: Fig. 3*B*, *Lanes 5* to *8*). A third Lyb-2^b lysate was precleared twice with B9.689 and then divided in half and reprecipitated with Lyb-2.2-specific antiserum or K10.6 (reduced: Fig. 3*A*, *Lanes 10* to *13*; nonreduced: Fig. 3*B*, *Lanes 9* to *12*). After preclearing NB2-Lyb-2^b lysates twice with Lyb-2.2-specific antiserum, K10.6, or B9.689, no protein band could be detected upon reprecipitation with the described Ab (reduced: Fig. 3*A*, *Lanes 4, 5, 8, 9, 12,* and *13*; nonreduced: Fig. 3*B, Lanes 3, 4, 7, 8, 11,* and *12*). Based on these data, we conclude that the Ly-19.2-specific mAb K10.6 recognizes a common epitope on the 45-kDa Lyb-2^a and Lyb-2^b polypeptides, whereas Ly-32.2-specific mAb B9.689 recognizes a unique epitope present on the 45-kDa Lyb-2^b polypeptide.

Discussion

The Ly-19.2 and Ly-32.2 alloantigens have been shown to map to the same region on mouse chromosome 4 as Lyb-2/CD72 and to have a mouse strain distribution pattern similar to that of specific Lyb-2/CD72 alleles (14, 15). Previous immunoprecipitation studies demonstrated that the Ly-19.2-, Ly-32.2-, and Lyb-2/CD72-specific mAb recognize antigenic determinants on the same molecular complex consisting of a 45-kDa polypeptide and a larger 95-kDa polypeptide (20). Because cytotoxicity assays showed Ly-19.2 and Ly-32.2 specificities to be expressed on some T cells as well as B cells, Ly-19 and Ly-32 were thought to be distinct molecules from Lyb-2/CD72, which has only been described on mouse B cells (1, 2, 14, 15). Upon isolation of the Lyb-2^a, -2^b, and -2^c cDNA, we examined whether the Ly-19.2 specificity represents an epitope present on both the Lyb-2^a and Lyb-2^b polypeptides, whereas the Ly-32.2 specificity represents a unique epitope present on only the Lyb-2^b polypeptide. The following lines of evidence support the conclusion that Ly-19 and Ly-32 are in fact Lyb-2/CD72. 1) Ly-19.2-specific mAb K10.6 stains NB2-Lyb-2^a and -2^b cDNA transfectants, but not NB2-Lyb-2^c cDNA transfectants or untransfected NB2 cells (Fig. 1). Ly-32.2-specific mAb B9.689 stains NB2-Lyb-2^b cDNA transfectants, but not NB2-Lyb-2^a and -2^c cDNA transfectants or untransfected NB2 cells (Fig. 1). 2) The K10.6, B9.689, and Lyb-2/CD72-specific Ab immunoprecipitate comigrating proteins from these NB2 transfectants under reducing and nonreducing conditions (Figs. 2 and 3). 3) The Lyb-2/CD72 polypeptide could be precleared from lysates of NB2-Lyb-2^a transfectants by K10.6- and Lyb-2.1-specific mAb 10.1.D2 (which recognizes Lyb-2^a), and from lysates of NB2-Lyb-2^b transfectants by K10.6, B9.689, and Lyb-2.2-specific antiserum (which recognizes Lyb-2^b) (Figs. 2 and 3). From these data, we conclude that the Ly-19.2-specific mAb K10.6, Ly-32.2-specific mAb B9.689, and Lyb-2/CD72-specific Ab all recognize a biochemically identical 45-kDa polypeptide representing Lyb-2/CD72.

If the Ly-19.2 and Ly-32.2 alloantigens represent distinct molecules from Lyb-2/CD72, then the Ly-19 and Ly-32

molecules would have to posess the following properties to explain our findings. NB2 (a rat T cell line), Cos-7 (a monkey kidney cell line), and L cells (a mouse fibroblast cell line) would all have to constitutively express, or be induced by transfection of Lyb-2/CD72 to express, Ly-19 and Ly-32, because transfection of these cell lines with the appropriate Lyb-2/CD72 cDNA resulted in positive immunofluorescence staining with the Ly-19.2 and Ly-32.2-specific mAb. Given that untransfected NB2, Cos-7, and L cells were not recognized by the Ly-19.2 and Ly-32.2-specific mAb, the hypothetical Ly-19 and Ly-32 gene products could only be brought to the cell surface or form their recognizable antigenic determinants in the presence of Lyb-2^a and Lyb-2^b (in the case of Ly-19) or just Lyb-2^b (in the case of Ly-32). Furthermore, not only would mouse Lyb-2/CD72 have to be able to complex with monkey Ly-19 and Ly-32 in Cos-7 cells as well as rat Ly-19 and Ly-32 in NB2 cells, but the mouse Ly-19.2 and Ly-32.2 allele-specific mAb would also have to recognize the monkey and rat Ly-19 and Ly-32 molecules, respectively. In addition, the putative distinct Ly-19 and Ly-32 polypeptides from the mouse, rat, and monkey cells would all have to comigrate with mouse Lyb-2/CD72 and/or not label with surface iodination.

Using the above scenario to reconcile the data presented in this paper with the hypothesis that Ly-19 and Ly-32 exist as distinct molecules from Lyb-2/CD72 is unrealistic. Furthermore, we have data demonstrating that Lyb-2/CD72 is expressed in some splenic T cells derived from mouse strains expressing Lyb-2^b, but not (or at much lower levels) in mice expressing Lyb-2^a or Lyb-2^c W. H. Robinson, M. M. T. Landolfi, and J. R. Parnes, manuscript in preparation). These data reconcile the previously observed differences in the tissue distribution pattern of expression of Lyb-2/CD72 (expressed only in immature and mature B cells) as compared to Ly-19.2 and Ly-32.2 (expressed in B cells and a small subset of T cells) (1, 14, 15). The previously reported differences in the tissue distribution of expression of these molecules involved comparisons of Lyb-2/CD72 expression in mice expressing Lyb-2^a with Ly-19.2 and Ly-32.2 expression in mice expressing Lyb-2^b (for which no Lyb-2/CD72-specific mAb had been described). Although Ly-19.2-specific mAb recognizes both Lyb-2^a and -2^b, in analyzing the tissue distribution of Ly-19.2 expression, Tada et al. (14) only reported studies of tissues from mice expressing Lyb-2^b, and not Lyb-2^a. Thus, it was not realized that in mouse strains expressing Lyb-2^a, expression of Ly-19.2 is restricted to the Lyb-2/CD72-expressing B cell subset (M. M. T. Landolfi and J. R. Parnes, unpublished observations). Based on our results, there seems to be no difference in the tissue distribution of expression of Ly-19, Ly-32, and Lyb-2/CD72, supporting our conclusion that Ly-19 and Ly-32 are in fact Lyb-2/CD72.

We performed immunoprecipitation analysis on transfectants expressing Lyb-2/CD72 to examine the biochemical relationship between Ly-19.2, Ly-32.2, and Lyb-2/CD72. Immunoprecipitates from NB2 cells transfected with the mouse Lyb-2^b and -2^c cDNA, as well as from the B cell line CH12.LX expressing Lyb-2^b (data not shown), contained 45-kDa doublets under reducing conditions. In contrast, immunoprecipitates from NB2-Lyb-2^a cDNA transfectants, and Cos-7 cells transiently transfected with the Lyb-2^a cDNA (3), contain a single 45-kDa polypeptide. Nevertheless, immunoprecipitates from L cells transfected with the Lyb-2^a cDNA contain the 45-kDa doublet (data not shown). Previous studies have reported mouse Lyb-2/CD72 to exist as a single 45-kDa polypeptide even though some of the published gels were overexposed to the extent that such a small difference in size might not be detected (3, 20, 21). In both B cell lines and L cells transfected with human Lyb-2/CD72, the human Lyb-2/CD72 polypeptide exists as 40- and 42-kDa forms, with a significantly larger molecular mass difference between forms as compared to that seen in mice (23). Endoglycosidase F treatment of human Lyb-2/CD72 immunoprecipitates reduced the size of both the 40- and 42-kDa bands to the same extent, indicating that the differences between these polypeptides are not due to differential N-linked glycosylation (23). Because the 45-kDa polypeptide doublet representing mouse Lyb-2/CD72 is observed in cDNA transfectants, it cannot represent the protein products of the previously described differentially spliced forms of Lyb-2/CD72 mRNA (22). We believe that the smaller polypeptide band in the doublet may represent a proteolytic cleavage product of the larger polypeptide, a difference in O-linked glycosylation, or an artifact of the immunoprecipitation process.

Nonreduced immunoprecipitates from NB2-Lyb-2^b and -2^c cDNA transfectants as well as B6 spleen cells expressing Lyb-2^b and AKR spleen cells expressing Lyb-2^c using the Ly-19.2, Ly-32.2, and Lyb-2/CD72 specific Ab contain comigrating, indistinguishable protein complexes of 90 kDa (Fig. 3*B, Lanes 1, 5,* and *9*; Fig. 4*B, Lane 2*; immunoprecipitates from spleen cells not shown). These nonreduced immunoprecipitates also contain a high molecular mass smear of complexes greater than 200 kDa (Fig. 3*B, Lanes 1, 5,* and *9*; Fig. 4*B, Lane 2*; immunoprecipitates from spleen cells not shown). Lyb-2/CD72 contains a region resembling a leucine zipper (28), conserved in all three mouse alleles and between mice and humans (22, 23), which may enable the Lyb-2/CD72 polypeptides to form 90-kDa homodimers. This region in Lyb-2/CD72 lacks an adjacent basic region that could bind DNA, and thus differs from leucine zipper domains in transcription factors. Lyb-2/CD72 is a member of a subfamily of structurally related membrane-bound receptor proteins in the C-type lectin superfamily (3, 29). The leucine zipper-like regions in this subfamily form α-helical coiled-coil stalks that, based on

Table I
Allele and epitope specificities of mouse Lyb-2/CD72-specific mAb

Lyb-2/CD72 mAb	Previous Lyb-2 Epitope Designation[a]	Proposed CD72 Epitope Designation	Lyb-2/CD72 Alleles Recognized[b]		
			Cd72[a] (Lyb-2[a])	Cd72[b] (Lyb-2[b])	Cd72[c] (Lyb-2[c])
10.1.D2	Lyb-2.1	CD72.1	+	–	–
B9.689	Not defined[c]	CD72.2	–	+	–
K10.6	Not defined[d]	CD72.4	+	+	–
82–9.2	Lyb-2.3	CD72.3	–	–	+

[a] Lyb-2 epitopes designated by Tung et al. (17) based on spleen cell cytotoxicity assays using 10.1.D2, 82–9.2, and Lyb-2 allele-specific antisera.
[b] Shen et al. (16) identified the Lyb-2[a], -2[b], and -2[c] Lyb-2/CD72 alleles based on spleen cell cytotoxicity assays using antisera generated by cross-mouse strain spleen cell immunization.
[c] The same or a similar epitope to the Lyb-2.2 epitope defined by Tung et al. (17).
[d] The same or a similar epitope to the Lyb-2.4 epitope defined by Tung et al. (17).

experimental evidence, exist as homodimers or homotrimers (30). We believe that the 90-kDa protein complex present in our nonreduced immunoprecipitates of Lyb-2[b] and -2[c] from both transfectants and spleen cells represents disulfide-linked homodimers of Lyb-2/CD72, and that this is likely to be the predominant form of Lyb-2/CD72 expressed on the cell surface. Due to the 15 conserved cysteine residues among the mouse Lyb-2/CD72 alleles (22), it is possible that the 95-, 100-, or 105-kDa bands observed in some Lyb-2/CD72 immunoprecipitates under reducing conditions may be due to artifactual formation of disulfide-bridges between Lyb-2/CD72 and other proteins during the immunoprecipitation procedure. It is also possible that the 95-, 100-, or 105-kDa bands could represent a B cell-specific polypeptide that physiologically associates with the Lyb-2/CD72 complex. Formation of artifactual disulfide linkages between Lyb-2/CD72 and other proteins is one possible explanation for the observation by Nakamura et al. (20) that nonreduced immunoprecipitates using Ly-19.2-, Ly-32.2-, and Lyb-2/CD72-specific Ab would not enter their stacking gels, and for the marked variation in the relative amounts of this higher molecular mass band seen under reducing conditions in different studies. This could also explain the smear present in our nonreduced Lyb-2[a] immunoprecipitates (data not shown) as well as our observation that a significant amount of nonreduced Lyb-2/CD72 immunoprecipitates did not enter the stacking or separating gels. In contrast, in human Lyb-2/CD72 immunoprecipitates, the majority of the nonreduced sample enters the separating gel and yields a major band of 86 kDa (and a minor band of 92 kDa), implying that human Lyb-2/CD72 exists on the cell surface as a disulfide-linked homodimer (23). This is consistent with the presence of 90-kDa protein complexes representing disulfide-linked homodimers of mouse Lyb-2/CD72 in some of our nonreducing immunoprecipitates. However, we cannot eliminate the possibility that homotrimers or other higher molecular mass complexes involving Lyb-2/CD72 (and potentially other proteins) are present.

To help clarify the specificities of the various mAb recognizing mouse Lyb-2/CD72, we have modified the system originally proposed by Tung et al. (17) to adapt it to the CD nomenclature and to include the newly identified Lyb-2/CD72-specific mAb K10.6 (which recognizes Lyb-2[a] and -2[b]) and B9.689 (which recognizes Lyb-2[b]). von Hoegen et al. (24) established that the human Lyb-2 cDNA (23) encodes a molecule biochemically identical to that recognized by human CD72-specific mAb. Our classification system for mouse Lyb-2/CD72 is presented in Table I and is derived from our cell surface staining and immunoprecipitation data presented both in this paper and by Robinson et al. (22).

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