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Cloning of BY55, a Novel Ig Superfamily Member Expressed on NK Cells, CTL, and Intestinal Intraepithelial Lymphocytes[1]

Anukanth Anumanthan,* Armand Bensussan,† Laurence Boumsell,† Andreas D. Christ,‡ Richard S. Blumberg,‡ Stephan D. Voss,* Amish T. Patel,* Michael J. Robertson,* Lee M. Nadler,* and Gordon J. Freeman[2]*

Expression of the BY55 protein has been shown to be tightly associated with NK and $CD8^+$ T lymphocytes with cytolytic effector activity. To determine the function of this protein, we molecularly cloned BY55 cDNA. The cDNA sequence predicts a cysteine-rich, glycosylphosphatidylinositol-anchored protein of 181 amino acids with a single Ig-like domain weakly homologous to killer inhibitory receptors. Reduction and carboxyamidomethylation of immunoprecipitated BY55 gave a band of 27 kDa, whereas reduction alone led to an 80-kDa species, suggesting that BY55 is a tightly disulfide-linked multimer. RNA blot analysis revealed BY55 mRNAs of 1.5 and 1.6 kb whose expression was highly restricted to NK and T cells. BY55 was expressed on the $CD56^{dim}$, $CD16^+$ subset of NK cells, which have high cytolytic activity, but was not expressed and was not induced on the $CD56^{bright}$, $CD16^-$ subset of NK cells, a subset with high proliferative, but low cytolytic, capacity. In human tissues, BY55 mRNA was expressed only in spleen, PBL, and small intestine (in gut lymphocytes). BY55 was expressed on all intestinal intraepithelial lymphocytes, which were predominantly $CD3^+TCR\alpha/\beta^+CD4^-CD8^+CD11b^+CD28^-CD45RO^+CD56^-CD101^+CD103^+$ ($\alpha_E\beta_7$ integrin). In addition, BY55 was expressed on most $CD8^+CD28^-$ peripheral blood T cells. These phenotypic relationships suggest that $CD8^+CD28^+$ precursor CTL may terminally differentiate into $CD8^+CD28^-BY55^+$ effector CTL and that some of the peripheral blood $CD8^+CD28^-$ subset may represent recirculation from mucosal epithelial immune sites. ***The Journal of Immunology*****, 1998, 161: 2780–2790.**

Cytotoxic lymphocytes are responsible for the lysis of virally infected target cells and defense against tumor cells (1). CTL perform their lytic activity after specific recognition of antigenic peptide embedded in MHC class I molecules. In contrast, NK lymphocytes are inhibited in their cytotoxic activity after recognition of self-MHC class I molecules (2–5). Both lymphocyte types use the same lytic machinery, consisting of proteolytic enzymes including granzymes and perforins (1, 6–8). Enumeration of CTL is only possible using a lengthy precursor CTL assay. Enumeration of NK cells has relied on phenotypic studies with CD56 (9), CD16 (10), and CD57 (11). More recently, NK cell identification has been performed using various Abs to their MHC binding receptors collectively referred to as killer inhibitory receptors (KIR)[3] (reviewed in Refs. 12–15). However, the KIR are neither expressed by the whole NK cell population nor predict their effector cytotoxic activity. In addition, the expression of KIR is not restricted to NK cells.

The BY55 mAb was developed following immunization with YT2C2, a human leukemia cell line with NK activity, to further delineate lymphocytes with cytotoxic activity (16). The BY55 mAb was shown to be unique and to immunoprecipitate an 80-kDa cell surface structure (16). Of the greatest importance, the BY55 mAb stains all lymphocytes exhibiting cytotoxic activity (16, 17). Depletion of $BY55^+$ lymphocytes removed both NK lytic activity and CTL activity (16–18). BY55 mAb bound to only 10 to 25% of E-rosette$^+$ PBLs and not to normal B or myeloid cells. The BY55 mAb recognizes most lymphocytes with an NK-like phenotype ($CD16^+$, $CD56^+$) and a sizable proportion of $CD3^+CD8^+$ T cells with $TCR\alpha/\beta^+$ or γ/δ^+, but not $CD4^+$ T cells (16, 17). BY55 expression was lost from the cell surface within 1 h following in vitro activation of E^+ PBLs with PMA, and BY55 expression did not reappear even after 3 days of culture (16). In cord blood, all $BY55^+$ lymphocytes have an NK-like phenotype, and $BY55^+$ $CD8^+$ CTL are not yet present (18). In HIV-infected individuals, the number of $CD8^+$ T lymphocytes increased by twofold compared with that in normal individuals, but the number of $BY55^+CD8^+$ T cells was increased even further (fourfold), suggesting a role for $BY55^+$ CTL in the response to HIV (18). In contrast, the $BY55^+$ NK cell population was decreased following HIV infection.

To further study the BY55 molecular structure, we cloned the gene encoding BY55 by COS cell expression cloning. The cDNA sequence predicts a cysteine-rich, glycosylphosphatidylinositol (GPI)-anchored protein of 181 amino acids with a single Ig-like domain weakly homologous to KIR. Further, RNA blot analysis confirmed that BY55 expression is highly restricted to NK and T cells. BY55 was expressed on the $CD56^{dim}$, $CD16^+$ subset of NK

*Department of Adult Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115; †Institut National de la Santé et de la Recherche Médicale, Unit 448, Faculte de Medicine de Creteil, Paris, France; and ‡Gastroenterology Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

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[2] Address correspondence and reprint requests to Dr. Gordon J. Freeman, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115. E-mail address: gordon_freeman@macmailgw.dfci.harvard.edu

[3] Abbreviations used in this paper: KIR, killer inhibitory receptors; GPI, glycosylphosphatidylinositol; iIEL, intestinal intraepithelial lymphocytes; ECL, enhanced chemiluminescence; PIPLC, phosphatidylinositol-specific phospholipase C; LCM, leukocyte-conditioned medium; CRRY, complement regulatory protein.

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cells, which have high cytolytic activity, but was not expressed on the $CD56^{bright}$, $CD16^-$ subset of NK cells, a subset with high proliferative but low cytolytic capacity. BY55 protein was expressed on all intestinal intraepithelial lymphocytes (iIEL) which are $CD8^+CD28^-CD11b^+$ cells, similar to the subset of $BY55^+CD8^+CD28^-CD11b^+$ peripheral blood T cells. Overall, our results further demonstrate that the BY55 cell surface molecule delineates functional cytotoxic lymphocytes. These phenotypic relationships suggest that $CD8^+CD28^+$ precursor CTL may terminally differentiate into $CD8^+CD28^-BY55^+$ effector CTL and that some of the peripheral blood $CD8^+CD28^-$ subset may represent recirculation from mucosal epithelial immune sites.

Materials and Methods

Antibodies

FITC- and PE-conjugated class- and subclass-specific goat anti-mouse Ig secondary Ab were purchased from Southern Biotechnology (Birmingham, AL). BY55 mAb was used at a 1/500 dilution of clarified ascitic fluid (16). Anti-CD8, 7PT 3F9, IgG2a; anti-CD11b, Mo1, IgM; anti-CD14: Mo2, IgM; anti-CD16, 3G8, IgG1; anti-CD19, B4, IgG1; anti-CD20: B1, IgG2a (19), anti-CD28, 9.3, IgG2a (20) (Dr. C. June, Naval Research Institute, Bethesda, MD); anti-MHC class II, 9-49, IgG2a; and anti-CD94, NKH3 (21), have been described previously. CD8 α/β-specific mAb 2T8-5H7 was provided by Dr. Ellis Reinherz (Dana-Farber Cancer Institute, Boston, MA). TCRα/β-specific Ab BMA031 and TCRγ/δ-specific Ab IMMU510 were obtained from Immunotech (Westbrook, ME). Anti-$\alpha_E\beta_7$ integrin (CD103) mAb ($\alpha_E\beta_7$-2, IgG1) (22) was provided by Dr. Gary Russell (Brigham and Women's Hospital, Boston, MA). Fluorochrome-conjugated NKH-1 (CD56, IgG1), CD16 (IgG1), and unconjugated control IgM were obtained from Coulter (Hialeah, FL). All other Abs were purchased from Coulter.

Phenotypic analysis

Expression of cell surface molecules was determined by direct or indirect labeling using standard methodology. Irrelevant isotype-matched Abs (IgG subclasses or IgM) were used as negative controls. FITC- or PE-conjugated goat anti-mouse IgG or IgM were used for unconjugated mAbs. Samples were analyzed in a Coulter Elite flow cytometer.

Chemicals

Protease inhibitors, DTT, iodoacetamide, and *n*-octyl-β-D-glucoside were obtained from Sigma (St. Louis, MO). Protein A-Sepharose 4B was purchased from Repligen (Cambridge, MA). ECL Western blot reagents were obtained from Amersham (Arlington Heights, IL). [α-^{32}P]dATP and -dCTP were obtained from New England Nuclear (Boston, MA).

COS cell expression cloning

A cDNA library was constructed in the pCDM8 vector using NK cell poly(A)$^+$ RNA pooled from four different donors. For the first round of selection, COS cells were transfected via the DEAE-dextran procedure (23) with 0.2 μg of NK plasmid library DNA/100-mm dish. After 40 h, cells were harvested, incubated with BY55 mAb (1/500 dilution of ascites), washed, and panned on anti-IgM-coated plates as previously described (23, 24). Episomal DNA was prepared from adherent cells, reintroduced into *Escherichia coli*, and transfected into COS cells by polyethylene glycol-mediated fusion of spheroplasts (23), and the panning with BY55 was repeated. Individual plasmid DNAs were transfected into COS cells via the DEAE-dextran procedure (4 μg/100-mm dish) and analyzed after 72 h for cell surface expression of BY55 Ag by indirect immunofluorescence and flow cytometry. Nine of ten plasmids showed cDNA inserts of two different sizes, 1.3 or 1.4 kb. COS cells transfected with the miniprep plasmid DNA from eight of these clones bound BY55 but not control mouse IgM.

DNA sequence analysis

Both strands of one clone from the 1.3- and 1.4-kb human BY55 cDNA insert groups and of mBY55 (Integrated molecular analysis of genomes and their expression consortium clone accession no. AA276726) were sequenced with synthetic oligonucleotide primers and dye-labeled terminator/Taq polymerase chemistry and analyzed with an automated fluorescent DNA sequencer (Applied Biosystems, Foster City, CA). The accession numbers for the human and mouse BY55 sequences reported in this paper are AF060981 and AF060982, respectively. Homology searches of GenBank and EST databases were performed with the gapped BLAST program (25). Sequence comparisons and signal cleavage predictions were generated with the GAP and Sig_cleave programs of the Genetics Computer Group (Madison, WI) using the default parameters.

Northern and Southern blot analyses

Poly(A)$^+$ RNAs were prepared as described from various cell lines, denatured with formaldehyde, electrophoresed, and blotted onto nitrocellulose (26). Human tissue Northern blots were obtained from Clontech (Palo Alto, CA). The 1.3-kb BY55 cDNA was labeled by random oligonucleotide priming using α-^{32}P-labeled dCTP and dATP and a random labeling kit following the protocol recommended by the supplier (Boehringer Mannheim, Indianapolis, IN). Hybridization, washing, and autoradiography were performed as previously described (27).

Stable transfection

CHO and 300.19 cells were stably transfected by electroporation with linearized BY55 plasmid DNA and the drug selection plasmids pGK-hygromycin or pSV2-neomycin, respectively. Following drug selection, transfected cells expressing BY55 Ag were selected by FACS sorting with BY55 mAb, and single cells expressing BY55 were cloned by limiting dilution.

Cell surface biotinylation and immunoprecipitation

Cells were biotinylated by a sulfosuccinimidobiotin (Sulfo-NHS-biotin, Pierce, Rockford, IL) procedure. Briefly, after three washes in PBS, cells were suspended at 10×10^6/ml in PBS with 0.1 M HEPES (pH 8.0) and 0.1 μg/ml of Sulfo-NHS-biotin. After a 40-min incubation at room temperature with occasional shaking, cells were washed three times with RPMI 1640 at 4°C. Cells were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 60 mM *n*-octyl-β-D-glucoside, and 0.05% Triton X-100 in the presence of the protease inhibitors aprotinin (1 U/ml), benzamidine hydrochloride (10 μg/ml), leupeptin (1 μg/ml), pepstatin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), and PMSF (1 mM) by rocking at 4°C for 45 min. After centrifugation, the lysate supernatant was precleared first by incubation with protein A-Sepharose 4B beads and second by incubation with a control rabbit polyclonal antiserum plus protein A-Sepharose 4B. Immunoprecipitation was performed by an overnight incubation with BY55 or a control IgM Ab plus goat anti-mouse IgM coupled Sepharose-4B (Zymed Laboratories, South San Francisco, CA). After five washes with a wash buffer containing 50 mM Tris-HCl (pH 8.3), 0.5 M NaCl, and 0.5% Nonidet P-40, protein was either eluted directly by boiling for 10 min in SDS sample buffer containing 5 mM β-ME or alternatively was reduced with 10 mM DTT in wash buffer with a 1-h incubation at 60°C and carboxyamidomethylated with 100 mM iodoacetamide at 90°C, and the protein was eluted as before. The immunoprecipitates were resolved by 10% SDS-PAGE and blotted electrophoretically onto Immobilon membrane (Millipore, Bedford, MA). The membrane was blocked overnight with 5% dried milk in PBS plus 0.05% Tween-20, and the protein bands were developed with horseradish peroxidase-conjugated streptavidin and ECL reagents.

Phosphatidylinositol-specific phospholipase C (PIPLC) treatment

Cells were washed with cold RPMI three times, incubated with phosphatidylinositol-specific phospholipase C (0.4 U/ml) plus 1 mM PMSF for 1 h at 37°C, and washed again three times with cold RPMI. Monophosphatidylinositol-specific phospholipase C (recombinant grade) was obtained from Oxford Glyco System (Rosedale, NY). Control cells were treated similarly but without PIPLC. Cells were then stained with either BY55 or control Abs and PE-conjugated secondary Ab and analyzed by FACS.

Isolation of iIEL

The iIEL were purified essentially as described previously (28). Briefly, mucosa of surgically removed small intestinal specimen was separated from the submucosa by dissection. After washing several times with RPMI 1640, the mucosa was cut into 1-cm^2 pieces. Tissues were washed three times with HBSS (without calcium or magnesium) and 1 mM DTT by shaking in a water bath at 37°C for 30 min. The iIELs were then eluted from the tissue with 0.75 mM EDTA in HBSS (without calcium or magnesium) containing 10 mM HEPES (pH 7.4), penicillin, and streptomycin by incubating in a shaking water bath at 37°C for three separate 45-min periods. The eluted iIELs were pooled and washed in culture medium (RPMI 1640, 10% FCS, 2 mM glutamine, 1% nonessential amino acids, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10 mM HEPES, pH 7.4) and kept at 4°C overnight. The iIELs were further purified by centrifugation

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through a 40/60% Percoll gradient (Pharmacia, Piscataway, NJ) and isolation of the iIELs at the 40%/60% interface. Purified iIELs were then washed in RPMI 1640.

Isolation of $CD8^+CD28^-$ PBL

PBMC were isolated from fresh whole blood by centrifugation over Ficoll-Hypaque. Monocytes were depleted by adherence to plastic flasks for 2 h to overnight at 37°C. Cells were incubated with Abs specific for NK cells (anti-CD16 (3G8, IgG1) plus anti-CD56 (3B8, IgG1)), B cells (anti-CD19 (B4, IgG1) plus anti-CD20 (B1, IgG2a)), macrophages (anti-CD14 (Mo2, IgM)), $CD4^+$ T cells (anti-CD4 (T4, IgG1)), and $CD28^+$ T cells (anti-CD28 (9.3, IgG2a)) and NK cells, B cells, macrophages, $CD4^+$ T cells, and $CD28^+$ T cells were depleted using goat anti-mouse IgG and IgM Ab-coated magnetic beads (PerSeptive Diagnostics, Framingham, MA).

Isolation and culture of human NK cells

PBMC were isolated by Ficoll-diatrizoate density gradient centrifugation from cytophoresis buffy coats obtained from normal volunteer donors. Adherent mononuclear cells were depleted by incubation on sterile scrubbed nylon wool columns for 60 min at 37°C. Enriched NK cells were obtained by negative selection using T1/24T6G12 (CD5), T3/RW2 (CD3), and MY4 (CD14) mAb together with immunomagnetic beads as described previously (29). Highly purified $CD56^{bright}$ and $CD56^{dim}$ NK subsets were isolated from populations of enriched NK cells by cell sorting as previously described (30). Basal culture medium was RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml gentamicin, and 15% heat-inactivated FCS. Activation and in vitro expansion of NK cells using leukocyte-conditioned medium (LCM) and ionomycin were performed as previously described (31). Polyclonal NK cell cultures were maintained at cell concentrations of 1 to 2×10^6/ml by addition of basal medium supplemented with 10 to 15% LCM.

Proliferation assays

Sorted NK cells were plated at 30,000 cells/well (1.5×10^5 cells/ml) in 96-well microtiter plates (Flow Laboratories, McLean, VA) with the indicated concentrations of IL-2 and/or mAb. The final dilution of mAb-containing ascites added to the cultures was 1/500. In some experiments, sorted NK cells were cultured together with 5,000 irradiated (10,000 cGy) K562 stimulator cells at an NK to stimulator cell ratio of 5:1. Cells were cultured for 96 h, followed by a 16-h pulse with 1 μCi [^{3}H]thymidine.

Results

Molecular cloning and characterization of BY55

COS cell expression cloning using BY55 mAb and a cDNA library constructed from NK cell mRNA resulted in the isolation of 1.3- and 1.4-kb cDNAs. On transient transfection into COS cells, both the 1.3- and 1.4-kb cDNAs directed the expression of a cell surface protein that was recognized by the BY55 mAb, but not by isotype-matched control IgM (Fig. 1).

Sequencing of the 1.3- and 1.4-kb BY55 cDNAs generated sequences of 1425 and 1319 nucleotides. Sequence analysis of the two clones revealed an identical single long open reading frame of 181 amino acids. The two cDNA transcripts differed only in the 1.4-kb cDNA having an extra 106 nucleotides in the 5′ untranslated region. This extra sequence is a repetitive sequence bounded by splice donor/acceptor sites and is presumably spliced out to produce the 1.3-kb cDNA. DNA database searches identified no related human sequences and a highly homologous murine EST (GenBank accession no. AA276726). Complete sequencing of this murine EST showed it to be the murine BY55 homologue with 65% amino acid identity, and Figure 2*A* shows a comparison of the predicted amino acid sequences of human and murine BY55. BY55 has two hydrophobic domains, one at the amino terminus and the other at the carboxyl terminus (Fig. 2*B*). The amino terminal hydrophobic domain (human, amino acids 1–25) has the characteristics of a secretory signal sequence with a predicted cleavage site after the glycine at position 25. The carboxyl-terminal hydrophobic domain comprises the last 15 (human) or 18 (mouse) amino acids of the open reading frame. This is shorter

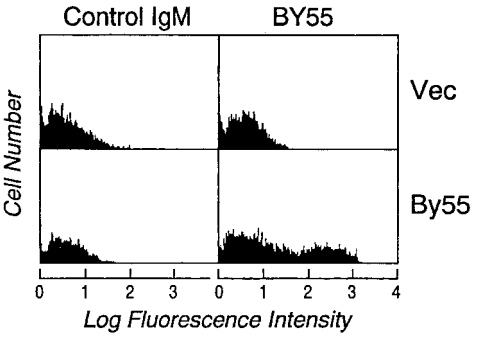

FIGURE 1. BY55 Ab binds to COS cells transfected with BY55 cDNA. COS cells were transfected with pCDM8 vector alone (Vec) or with BY55 cDNA in pCDM8 (BY55), as indicated at the *right*. Transfectants were stained with either BY55 mAb (BY55) or isotype-matched control Ab (control IgM) as indicated *above the panel*. Ab binding was detected by indirect immunofluorescence with PE-conjugated anti-IgM antisera and flow cytometric analysis.

than expected for a membrane anchor and is not followed by charged amino acids as is usual for transmembrane domains, but is characteristic of a GPI membrane anchor signal sequence (32). BY55 and GPI-anchored human CD58 (LFA-3) and rat CD48 share the sequence SSG at position −6 from the carboxyl-terminal hydrophobic domain with the GPI linkage predicted to be added to the first serine at position 159, following cleavage after these amino acids. Thus the mature, unmodified human BY55 polypeptide would have 134 amino acids and a predicted m.w. of 14,901. The BY55 polypeptide has two (human) or three (mouse) potential sites for *N*-linked glycosylation. Surprisingly, the hydrophobic signal and carboxyl domains are more conserved (68 and 86%, respectively) than the mature extracellular domain (62%). This may indicate that the hydrophobic signal and carboxyl domains contain important information for intracellular sorting or cell surface localization.

Structural analysis showed that the BY55 extracellular domain was comprised of a single Ig-like domain containing amino acids characteristic of an Ig superfamily member and confirmed the presence of alternating regions of β-sheet structure characteristic of Ig domains (data not shown). Protein database searches revealed homology to Ig-V domains of the pregnancy-specific glycoprotein family (33) and the first Ig-C2 domain of KIR. This homology is weak, with 29% identity (44% similarity) between BY55 and the KIR2DL4 subset of KIR (34, 35) (Fig. 2*C*). The 68-amino acid distance between the Ig cysteines at positions 44 and 112 is more characteristic of an Ig-V domain than an Ig-C domain. The BY55 structure is unusual for an Ig superfamily member in having an additional cysteine immediately following the second Ig cysteine. This dicysteine is found only in three λ Ig-V domains and one other Ig superfamily member, IGSF1 (accession no. AF034198). BY55 also has two cysteines between the Ig cysteines, potentially looping out a short region between amino acids 61 and 68. This unusual loop is found in only a few Ig superfamily members, such as CTLA4 and polymeric Ig receptor. In summary, BY55 is cysteine rich, with six cysteines in the mature polypeptide, including the first amino acid of the predicted mature polypeptide, and is composed of a single Ig-like domain. Thus, BY55 has ample capacity for forming intra- and interchain disulfide bonds.

Expression of BY55 mRNA

Two mRNA transcripts of 1.5 and 1.6 kb were identified by hybridization with BY55 cDNA (Fig. 3), suggesting that the 1.3- and

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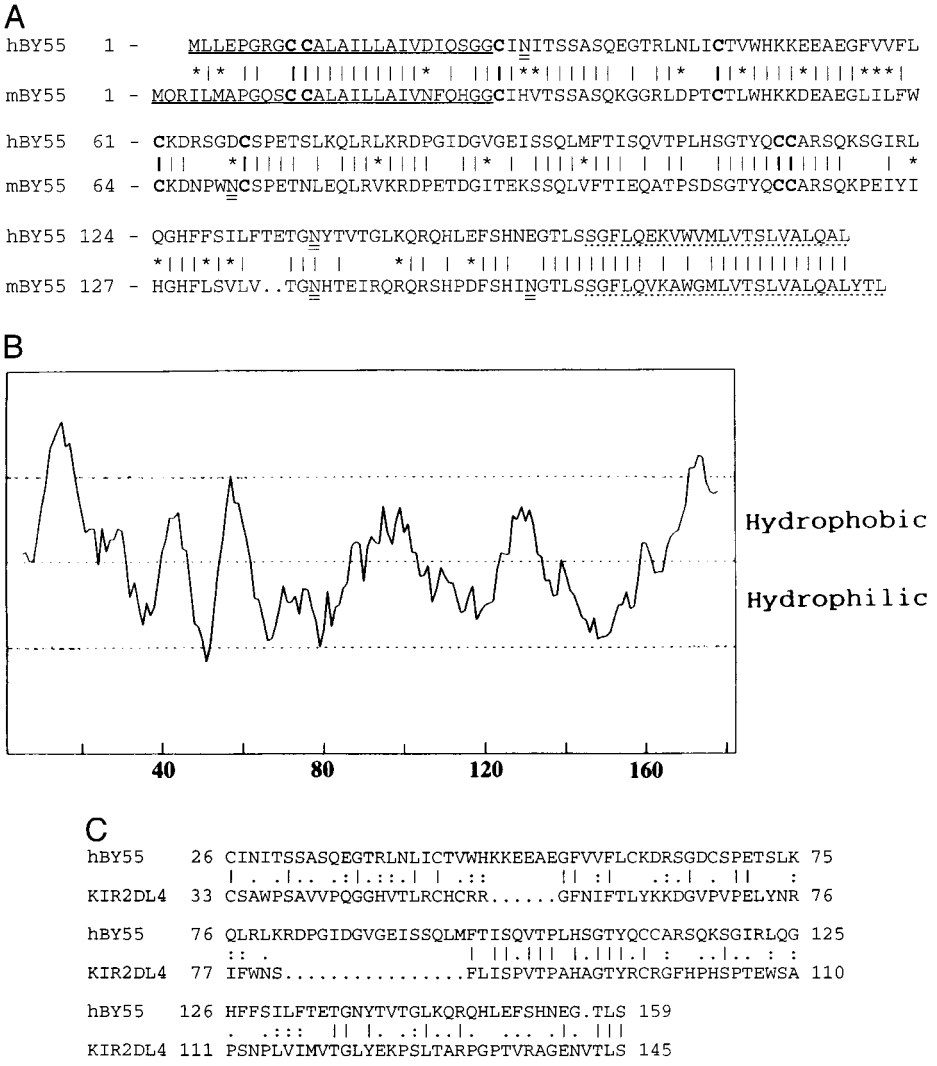

FIGURE 2. Predicted amino acid sequences of human and murine BY55. *A*, The amino acids predicted to form the signal sequence are singly underlined, and those for GPI anchorage are underlined with a dotted line. Potential sites for *N*-linked glycosylation are doubly underlined. The cysteines are in bold face. Identical amino acids are indicated by a vertical line, and conservative substitutions are shown by an asterisk. *B*, Chou-Fasman hydrophobicity index of the human BY55 open reading frame. *C*, Amino acid sequence of mature BY55 compared with KIR2DL4 (accession no. AF034773). | indicates identity, and : indicates similarity.

1.4-kb cDNAs, along with poly(A) tails, represent full-length cDNAs. Northern blot analysis of T, B, NK, and myeloid cell lines showed that BY55 mRNA was detected only in human NK cells and NKL, an NK-like cell line (36). BY55 mRNA was not expressed in Raji, a Burkitt's lymphoma B cell line; LBL-DR7, a lymphoblastoid B cell line; NALM6, a pre-B cell line; the T cell leukemia cell lines Jurkat, Rex, CEM, HPB-ALL, and Peer4; the HTLV-I transformed T cell lines SPP, MT2, and H9; or the myeloid cell line U937 (Fig. 3). In human tissues, BY55 mRNA was expressed only in spleen, PBL, and small intestine. As described below, BY55 expression in small intestine is in gut-associated lymphocytes. BY55 mRNA was not expressed in thymus, prostate, testis, ovary, heart, brain, placenta, lung, liver, skeletal muscle, kidney, or pancreas (Fig. 4, *A* and *B*). BY55 mRNA was not detectable in colon; however, the amount of RNA in this lane was less, as judged by the weak intensity of the control hybridization to the housekeeping gene G6PD.

Immunoprecipitation analysis

BY55 mAb has been shown to immunoprecipitate an 80-kDa protein from YT2C2, an NK-like cell line (16). Initial immunoprecipitation experiments using BY55 mAb and NKL cells, BY55-transfected CHO cells, or BY55-transfected 300.19 (a murine B cell line) also identified an 80-kDa polypeptide (Fig. 5, *lane 1*, and data not shown). In addition, there was a faint amount of a 27-kDa polypeptide. The size predicted for the mature BY55 polypeptide plus two *N*-linked glycosylations would be approximately 25 kDa. Thus, the observed 27-kDa band is close to the predicted size but the 80-kDa band is far larger than expected. Since BY55 protein has six cysteines in the mature polypeptide, there is ample possibility for the formation of intra- and interchain disulfide bonds that might be resistant to reduction or reform during electrophoresis (37) and increase the apparent m.w. We tested this hypothesis by reducing the BY55 immunoprecipitate with DTT, carboxyamidomethylating it with iodoacetamide, and resolving it by SDS-PAGE (Fig. 5, *lanes 2–6*). Following reduction and carboxyamidomethylation, the relative amount of the 27-kDa band was greatly increased relative to that of the 80-kDa band in BY55 immunoprecipitates from NKL cells and BY55-transfected 300.19 cells. Nevertheless, some 80-kDa band remained in both. These data are most consistent with BY55 being a tightly disulfide cross-linked multimer. BY55 is currently defined by a single IgM mAb, and definitive protein analysis must await the development of high affinity IgG mAbs.

BY55 is GPI anchored

To test whether BY55 is anchored to the membrane by a glycosylphosphatidylinositol linkage, we incubated NKL cells and BY55-transfected CHO cells with PIPLC and then analyzed cells for the expression of BY55 on the cell surface. PIPLC cleaved

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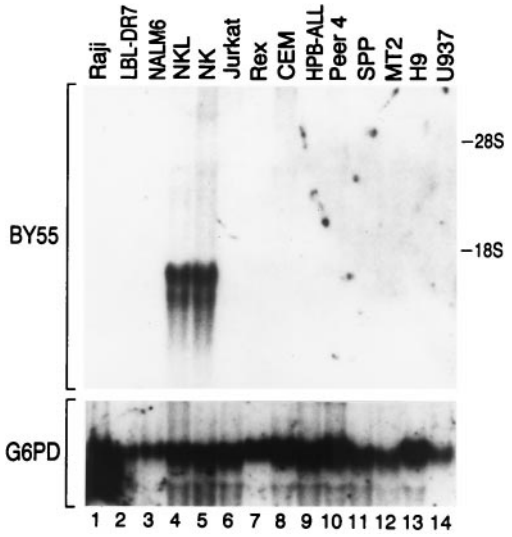

FIGURE 3. BY55 mRNA expression in T, B, NK, and myeloid cell lines. Each lane was loaded with 20 μg of total RNA from the indicated cell lines and probed with the 1.3-kb BY55 cDNA. The mRNA expression of the housekeeping gene G6PD is shown at the *bottom*.

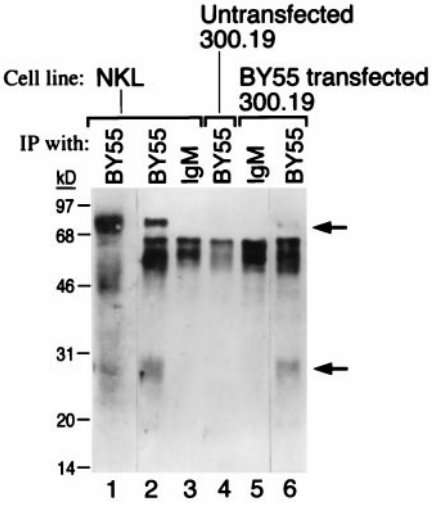

FIGURE 5. Immunoprecipitation analysis of BY55 protein. Cells were biotin labeled, and proteins were immunoprecipitated with BY55 mAb or an isotype-matched control Ab. Immunoprecipitates were denatured with SDS sample buffer containing β-ME, and in *lanes 2* to *6*, immunoprecipitates were first reduced with DTT and carboxyamidomethylated. Immunoprecipitates were resolved in a 10% SDS-PAGE gel and Western blotted, and the blot was developed with ECL detection reagents. Immunoprecipitates from 1) NKL with BY55 mAb; 2) NKL with BY55 mAb; 3) NKL with IgM; 4) untransfected 300.19, a murine pre-B cell line, with BY55 mAb; 5) BY55 transfected 300.19 with IgM, and 6) BY55 transfected 300.19 with BY55 mAb.

BY55 protein from the cell surface of NKL cells (Fig. 6, *top panel*) and BY55-transfected CHO cells (Fig. 6, *second panel*). The level of cleavage was almost 100% as opposed to some other GPI-anchored proteins that are incompletely removed by PIPLC (38, 39). As controls for PIPLC activity and the absence of protease activity, PIPLC was shown to cleave GPI-anchored hamster CD87 but not membrane-anchored complement regulatory protein (CRRY; Fig. 6, *bottom panels*).

BY55 is expressed on all intestinal intraepithelial lymphocytes

As shown in Figure 4, BY55 mRNA was expressed at high levels in the small intestine. As the small intestine has abundant gut-associated lymphoid tissue, we stained freshly isolated human small iIEL with BY55 mAb. Strikingly, BY55 was expressed on almost all iIEL (Fig. 7). The iIEL are a unique subpopulation of lymphocytes lining the gut, and almost all are $CD3^+TCR\alpha/\beta^+CD56^-$ (Fig. 7, *top panel*). For comparison, we stained PBL. Consistent with previous reports (16, 18), only 21% of peripheral blood $CD3^+$ T cells expressed BY55. Most peripheral blood NK cells expressed BY55, with 59% of $CD56^+$ PBL expressing BY55.

More extensive phenotypic analysis of iIEL revealed other characteristic markers expressed on this T cell subset. In addition to being predominantly $CD3^+TCR\alpha/\beta^+CD4^-CD8^+CD28^-CD56^-$, iIEL were almost all positive for $\alpha_E\beta_7$ integrin (CD103), CD11b (complement receptor type 3; data not shown), CD45RO, CD101, and the α/β CD8 heterodimer (Fig. 8).

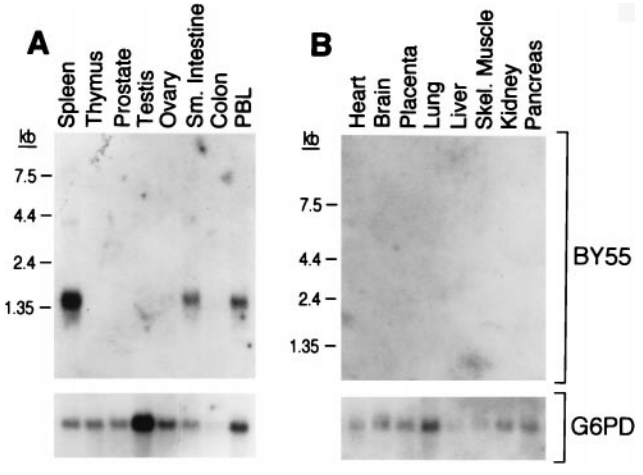

FIGURE 4. BY55 mRNA expression in human tissues. Expression of BY55 in human tissue Northern blots containing *A*) spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood lymphocytes; and *B*) heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Each lane contains 2 μg of poly(A)$^+$ RNA and was probed with the 1.3-kb BY55 cDNA. The positions of RNA m.w. markers are given on the left in kilobases. The mRNA expression of the housekeeping gene glyceraldehyde-6-phosphate dehydrogenase (G6PD) is shown at the *bottom* of each panel.

The subset of peripheral blood $CD8^+CD28^-$ T lymphocytes expressing BY55 differs from iIEL

The phenotype of iIEL, specifically that they were $CD8^+CD28^-CD11b^+$, is similar to a subset consisting of about half the peripheral blood $CD8^+$ T cells (40). To compare these populations, $CD8^+CD28^-$ peripheral blood T cells were prepared by depletion of macrophages and NK, B, and CD4- and CD28-positive cells. The $CD8^+CD28^-$ peripheral blood T cells uniformly expressed BY55 and CD11b but lacked $\alpha_E\beta_7$ and CD101 (Fig. 9 and data not shown). Expression of $\alpha_E\beta_7$ is induced by TGF-β and so may be a consequence of the intestinal microenvironment. Thus, iIEL share some, but not all, markers with the $CD8^+CD28^-$ peripheral blood T cell subset. This confirms previous work showing that in PBL the $CD3^+CD8^+$ subset, which does not express CD28, is predominantly $BY55^+$ (41). We have also reported that most $CD101^+$ T cells in peripheral blood are $CD28^+$ (41, 42).

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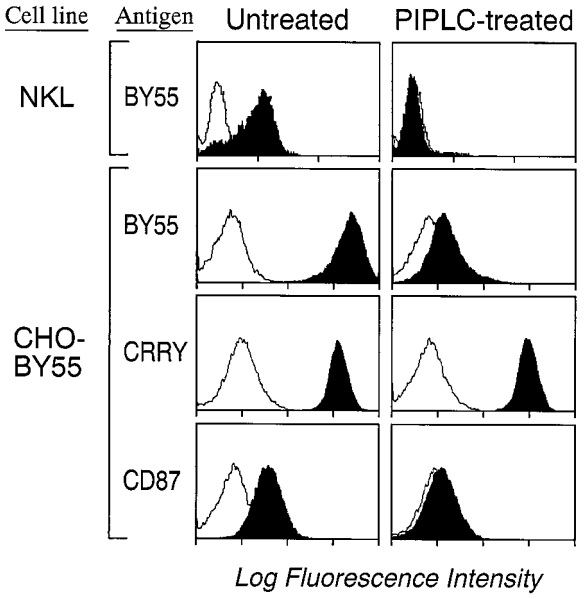

FIGURE 6. BY55 protein is anchored to the cell surface via a GPI linkage. *Top panel*, NKL cell line that expresses BY55. *Bottom panels*, BY55-CHO cell transfectants. Cells were either treated with PIPLC or untreated and were stained with either BY55 mAb or an isotype-matched control Ab followed by PE-conjugated goat anti-mouse IgM secondary Ab as indicated. As a control for PIPLC activity and protease contamination, CHO cells were also stained with mouse mAbs against hamster CD87 (urokinase receptor), which is GPI anchored, and hamster complement regulatory protein (CRRY), which is membrane anchored by a hydrophobic amino acid sequence. Data are presented as single color fluorescence histograms plotted with cell number vs log scale of fluorescence intensity. The indicated test mAbs are shown as shaded histograms, and isotype control Abs are shown as unshaded histograms.

The BY55$^+$ subset of blood NK lymphocytes expresses low levels of CD56 and is not proliferative

Human CD56$^+$ NK cells can be divided into CD16$^+$CD56^{dim} and CD16$^-$CD56^{bright} subsets (9, 30, 31, 38, 43) (Fig. 10). The CD16$^+$CD56^{dim} NK cell subset is highly cytolytic but does not proliferate well in response to known signals that costimulate NK cell proliferation (31, 43). In contrast, the CD16$^-$CD56^{bright} NK cell subset proliferates vigorously in response to mitogenic cytokines and costimulatory signals; however, it shows little lytic activity (21, 31, 43). We examined the expression of BY55 in these NK cell subsets and found that BY55 was expressed by the CD16$^+$CD56^{dim} NK cell subset and was absent from the CD16$^-$CD56^{bright} NK cell subset (Fig. 10). This is consistent with our previous reports showing the association of BY55 with cytolytic lymphocytes (16, 17). Following in vitro activation and expansion with IL-2, sorted CD56^{bright} cells remained BY55$^-$. Sorted CD56^{dim} cells were still 50% BY55$^+$ after in vitro activation, and expression of BY55 paralleled that of CD16 (data not shown).

Sorted BY55$^+$ CD56^{dim} NK cells did not proliferate well in response to IL-2 alone or together with anti-CD94 mAb (Fig. 11). In contrast, sorted BY55$^-$CD56^{bright} NK cells proliferated vigorously in response to increasing doses of IL-2, and this was augmented by costimulatory signals provided by anti-CD94 mAb. BY55 mAb neither augmented nor inhibited the proliferative response of either CD56^{bright} or CD56^{dim} NK cell subsets. K562 cells can costimulate the proliferation of human NK cells in response to IL-2 (31). In other experiments, inclusion of BY55 had no effect on the proliferation of CD56^{dim} NK cells in response to IL-2 and irradiated K562 cells (data not shown). Following in vitro activation and expansion, reculture of sorted NK cell subsets with IL-2 in the presence of BY55 had no significant effect on the proliferative response of these activated NK cell subsets (data not shown). Consistent with previous reports (16, 17), the presence of BY55 mAb did not augment or inhibit NK cell lysis (data not shown). Taken together, these results strongly strengthen our previous observation that BY55 is expressed only by NK cytotoxic cells, which proliferate poorly in response to IL2 alone.

Discussion

In the present report we describe the molecular cloning, expression, and further biochemical and functional characterization of the BY55 molecule. BY55 has a single Ig-like domain and is unique in its nucleotide and amino acid sequences. The Ig-like domain was related to a number of Ig superfamily members, with the highest degree of homology being to the pregnancy-specific glycoprotein and KIR families. Each KIR is expressed by a subset of NK cells and inhibits NK lysis of cells expressing its specific MHC class I ligand (reviewed in Refs. 12–15). BY55 was most related to the KIR2DL4 family of KIR, whose MHC ligand has not yet been defined (34, 35). If BY55 recognizes MHC class I, it would not be expected to deliver an inhibitory signal as it lacks an immunoreceptor tyrosine-based inhibition motif. The pregnancy-specific glycoproteins are a large family of Ig-like proteins secreted at high

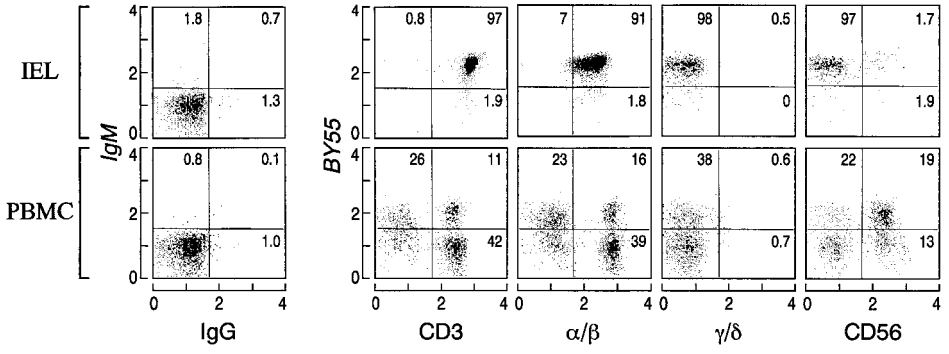

FIGURE 7. Human intestinal intraepithelial lymphocytes express BY55. Freshly isolated human small intestinal IELs or PBMC were incubated with PE-conjugated CD3 mAb (OKT3), TCRα/β mAb (BMA031), TCRγ/δ mAb (IMMU510), or CD56 mAb (NKH1) and with unconjugated BY55 mAb and FITC-conjugated goat anti-mouse IgM secondary Ab and analyzed by flow cytometry. Cells incubated with isotype-matched irrelevant Abs served as negative controls to set up quadrant regions. Data are presented as double color fluorescence histograms with log scale of fluorescence intensity. The percentage of cells positive in each quadrant is indicated within each quadrant.

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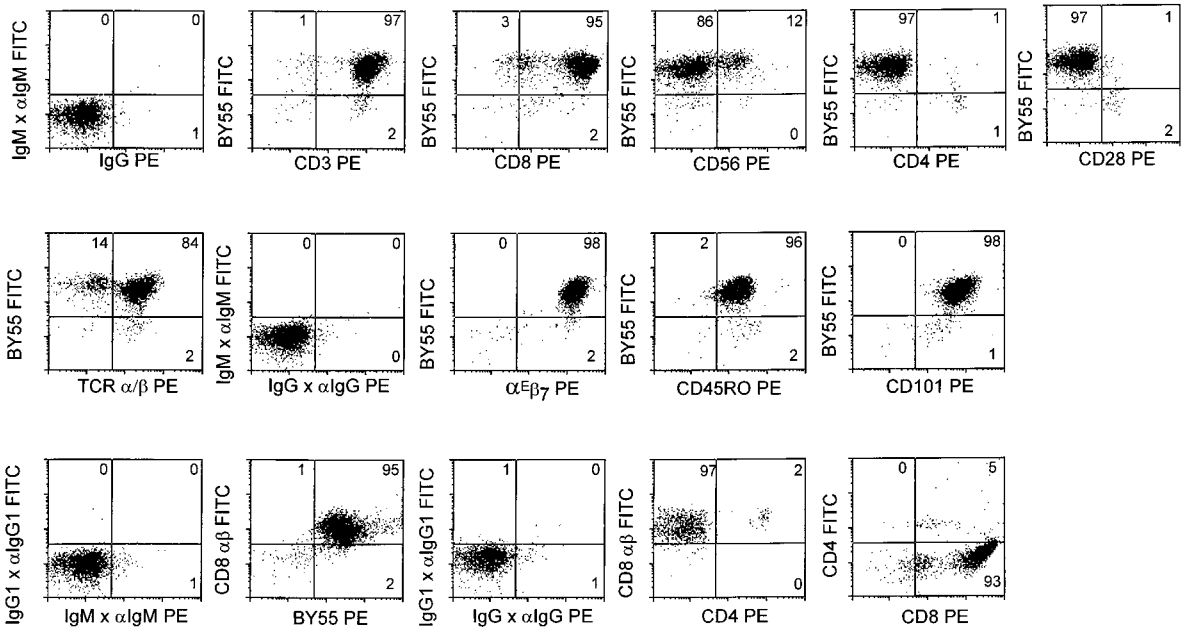

FIGURE 8. BY55 expression in iIEL. Freshly isolated human small intestinal IEL were incubated with FITC-conjugated CD3, CD4, CD8, CD28, TCRα/β, or CD56 mAbs or with unconjugated CD8 α/β, CD45RO, $\alpha_E\beta_7$, or CD101 mAbs followed by FITC-conjugated goat anti-mouse IgG secondary Ab. Cells were then stained with unconjugated BY55 mAb and PE-conjugated goat anti-mouse IgM secondary Ab and analyzed by flow cytometry. Cells incubated with isotype-matched irrelevant Abs served as negative controls to set up quadrant regions. The data are presented as double color fluorescence histograms with log scale of fluorescence intensity. The percentage of cells in each quadrant is indicated.

levels by the placenta (33). They are believed to play a role in tolerance of the fetus by the maternal immune system.

The BY55 cDNA encodes a polypeptide of 181 amino acids with two predicted *N*-linked glycosylation sites. Further, because the carboxyl-terminal hydrophobic domain was short and not bounded by charged residues, we investigated whether the BY55 molecule is anchored to the cell surface via GPI anchorage. Removal of BY55 protein from the cell surface by PIPLC treatment confirms that BY55 is indeed GPI linked. The BY55 carboxyl-terminal hydrophobic domain is novel, but the GPI addition site is similar to that of CD58. Gpi membrane anchorage of BY55 would allow rapid movement across the membrane, and cleavage of the GPI anchor would explain its rapid loss from the cell surface following PMA activation.

With the cleavage of both the secretory signal sequence of about 25 amino acids at the amino terminus and a GPI anchor signal sequence of about 22 amino acids at the COOH terminus, the resultant mature polypeptide would be 134 amino acids. We have previously reported that an 80-kDa protein was immunoprecipitated by BY55 mAb from cell surface iodinated cells (16). We show here that BY55 mAb also immunoprecipitates an 80-kDa protein from biotin-labeled NKL cells and BY55-transfected cell lines. This molecular mass is far larger than that predicted for mature BY55 polypeptide, with two *N*-linked glycosylations, approximately 25 kDa. Since BY55 protein has six cysteines in the mature polypeptide, there is ample possibility for forming intra- and interchain disulfide bonds, which might increase the apparent m.w. Reduction and alkylation of the immunoprecipitated BY55 gave rise to a band of 27 kDa, whereas reduction alone led to an 80-kDa species, suggesting that BY55 is a tightly disulfide-linked multimer. Moreover, we could eliminate the possibility that the 80-kDa predominant band immunoprecipitated by BY55 mAb is an associated chain similar to what has been described for CD94 associated protein (44), as this 80-kDa band was present in all immunoprecipitates, including those from BY55-transfected CHO cells and 300.19 murine B cells.

BY55 mRNA expression shows a remarkably restricted pattern. BY55 mRNA was expressed only in NK cells, NKL cells, spleen cells, and T cells, but not in any T, B, or myeloid cell line tested. In human tissues, BY55 mRNA expression also showed a very restricted distribution and was expressed only in spleen, small intestine, and peripheral blood, but not in thymus, prostate, testis,

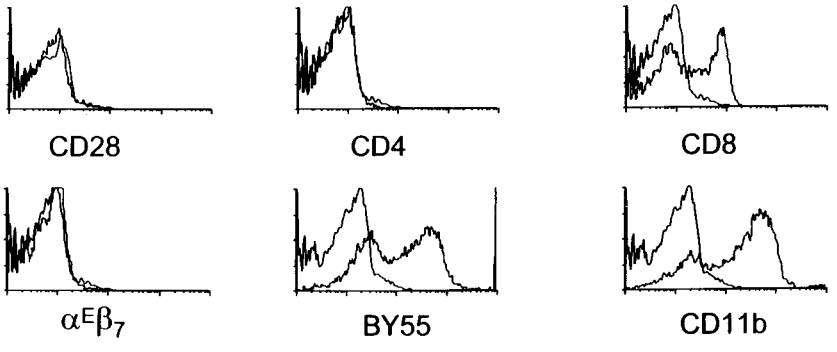

FIGURE 9. BY55 is expressed on $CD8^+CD28^-$ PBL. PBMC enriched for $CD8^+CD28^-$ cells were prepared as described in *Materials and Methods* and stained with the indicated mAbs (bold line) or isotype-matched negative controls (faint line).

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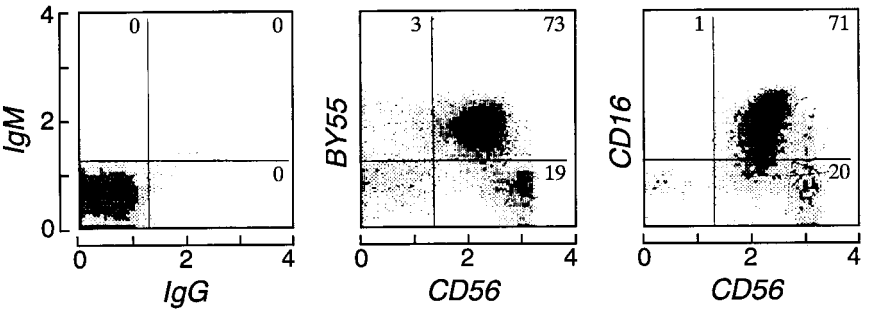

FIGURE 10. BY55 Expression in NK cell subsets. PBMC enriched for NK cells were prepared as described in *Materials and Methods* and stained with PE-conjugated CD56 (NKH-1) and either FITC-conjugated CD16 or unconjugated BY55 followed by secondary staining with FITC-labeled goat anti-mouse IgM. The logarithm of PE fluorescence is displayed on the abscissa, and the logarithm of FITC fluorescence is shown on the ordinate. The data shown are representative of three separate experiments.

ovary, heart, brain, placenta, lung, liver, skeletal muscle, kidney, or pancreas. The failure to detect BY55 expression in thymus may be a consequence of the low number of differentiated effector CTL in the thymus and suggests that further differentiative signals are required for BY55 expression in the periphery.

BY55 mRNA expression in human small intestine prompted us to investigate the cell surface phenotype of the lymphoid subsets isolated from small intestine. Two anatomically distinct lymphocyte populations are found in the intestinal mucosa, iIEL and lamina propria lymphocytes. Approximately one iIEL is present for every eight epithelial cells in the human small intestine, which, considering the vast area of the intestinal epithelium, makes the intestinal immune system by far the largest immune organ in the body. The iIELs in situ in nonpathologic conditions express some granzyme A, but not significant levels of Fas ligand, granzyme B, or perforin; however, they rapidly acquire cytolytic potential after in vitro cultivation with mitogens (45–47). Reports in mouse and man have indicated that IEL may function as cytolytic effector cells in pathologic conditions in vivo (48, 49). The iIEL have been shown to express immunoregulatory cytokines, such as IL-1β, IL-2, IL-8, IFN-γ, TNF-α, and TGF-β (45). The biologic functions of iIEL may include roles in defense against gut microorganisms, oral tolerance, and local immunosurveillance against epithelial cell injury and neoplastic transformation. The phenotypic properties of iIEL, $CD8^+CD45RO^+$ with a limited array of TCRα/β, suggests that they are memory T cells that recognize a limited number of Ags in the context of MHC class I or class I-like molecules (28, 46).

Interestingly, almost all the iIELs, which are $CD8^+$ TCRα/β T cells, expressed BY55. In addition, as previously reported, fresh iIELs expressed phenotypic markers of memory T cells such as CD45RO (50–52), $\alpha_E\beta_7$ integrin (CD103) (53), and CD101 (54), while they lacked the CD28 costimulatory receptor (54). The lack of expression of CD28 and the weak response to anti-CD3 cross-linking alone (55–57) suggest that they use alternative costimulatory pathways, perhaps through CD2 (55) or CD101 (54). We previously showed that $CD101^+$ cells represent a subset of peripheral blood $CD8^+CD28^+$ cells (41, 42). In addition, CD101 expression, similar to CD103 and CD45RO, is induced during activation. In contrast, we showed that BY55 is down-modulated after activation in short or long term culture (16). Besides the difference in phenotype between the cells expressing BY55 in PB and iIEL, both cell populations are able to exert cytotoxic function (17, 46, 57). This is in agreement with our earlier studies showing that in fresh PBL, only sorted $CD8^+BY55^+$ cells exhibit CTL activity in an anti-CD3-redirected lysis assay (17). Thus, it appears that BY55 delineates effector cytotoxic CTL in both circulating blood and intestinal tissue.

These results show that a subset of peripheral blood T cells has a striking phenotypic similarity to iIELs ($BY55^+$, $CD28^-$, $CD11b^+$, $CD8^+$, TCRα/β). Both iIELs and peripheral blood

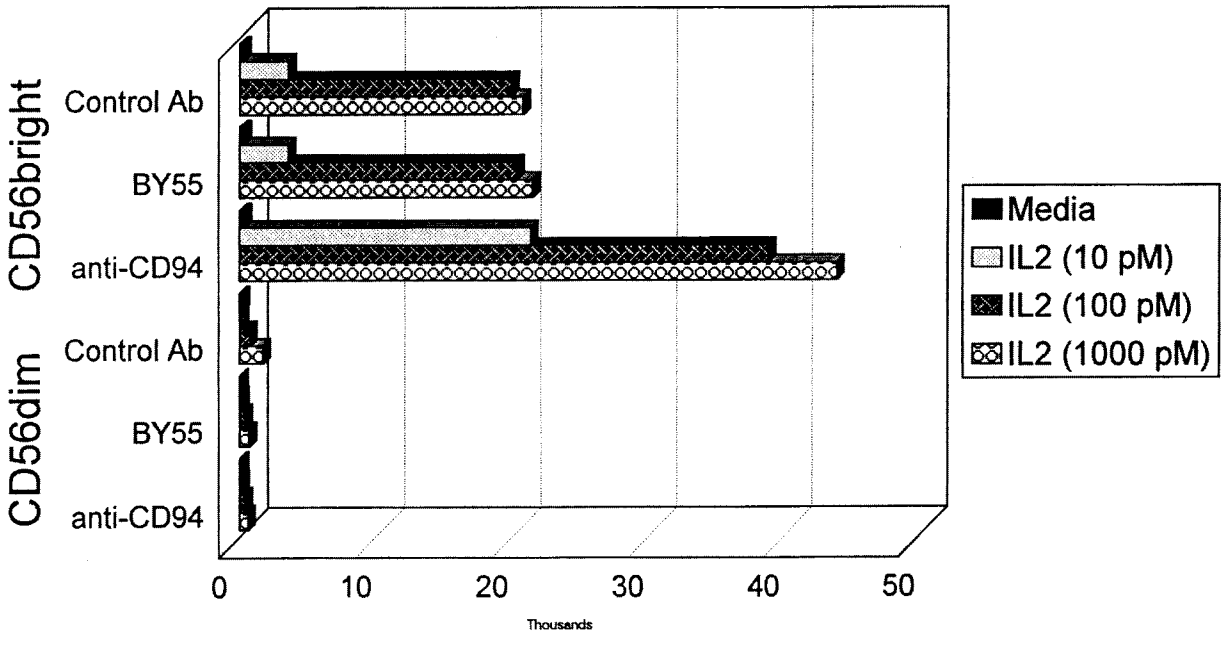

FIGURE 11. BY55 does not costimulate NK cell proliferation. Sorted $CD56^{bright}$ or $CD56^{dim}$ NK cells were incubated with IL-2 and the indicated mAbs, and proliferation was determined as described in *Materials and Methods*.

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$CD8^+28^-$ T cells have been shown to have an oligoclonal distribution of TCR β-chain usage (28, 58). We are critically testing the relationship between the blood and iIEL subsets by examining whether the oligoclonal distribution of TCR β-chain usage of iIEL is also found in peripheral blood $CD8^+28^-$ T cells. Similar TCR usage would validate assaying this blood $CD8^+28^-$ subset as a reflection of gut pathology.

BY55 was expressed on the $CD56^{dim}$, $CD16^+$ subset of NK cells, which have high cytolytic activity. In contrast, BY55 was not expressed and was not induced on the $CD56^{bright}$, $CD16^-$ subset of NK cells, a subset with high proliferative, but low cytolytic, capacity (31, 38, 43). Recently, several KIR/killer activating receptor structures defined as receptors for HLA class I molecules have been identified on NK cells and a subset of $CD8^+$ TCR^+ cells. These receptors have been shown to provide either inhibitory or activating signals following their ligation (reviewed in Refs. 12–15). Interestingly, the KIR/killer activating receptor can be coexpressed on the same NK cell, but they do not presently identify the whole circulating NK subset. BY55 is expressed on all the lytic NK cells, whereas each KIR is expressed on only a few percent of the total NK population. The homology of BY55 to the KIR suggests that it may have a role in NK target recognition. Nevertheless, the single BY55 mAb available does not inhibit or enhance allogeneic or NK cell lysis (16).

We show here that the $CD8^+28^-$ and $CD8^+BY55^+$ peripheral blood T cell populations are essentially the same. In HIV-infected individuals, $CD8^+$ T lymphocytes increased by twofold compared with those in normal individuals, and the level of $BY55^+CD8^+$ T cells was increased even further (fourfold), suggesting a role for $BY55^+$ CTL in the response to HIV (18). In contrast, the $BY55^+$ NK cell population was decreased following HIV infection. The $CD8^+28^-$ T cell subset contains the active anti-HIV cytolytic cells and high level IFN-γ producers (59–63). In contrast, the noncytotoxic antiviral activity that inhibits HIV replication is in the $CD8^+28^+$ subset (64). Progression of HIV infection leads to an expansion of the $CD8^+28^-$ T cell subset (65). During SIV infection in monkeys, the expanded population of blood $CD8^+28^-$ T cells has been shown to be cytolytic and to arise not from peripheral lymph node but from mucosal sites such as intestine and lung (66, 67). Thus, the expanded $BY55^+CD8^+$ T cell population in the peripheral blood of HIV-infected individuals probably represents recirculation from mucosal epithelial immune sites.

Abundant evidence shows that precursor cytolytic T cells require B7-mediated costimulation to develop into effector cytolytic cells (68, 69). This implies that the precursor cytolytic T cells must express CD28 to receive the costimulatory signal. Considerable evidence indicates that in vivo effector cytolytic T cells have lost CD28 expression (59–63). This implies a differentiative pathway in which precursor cytolytic $CD8^+CD28^+$ T cells receive a B7 costimulatory signal and develop into $CD8^+CD28^-$ effector cytolytic T cells. Azuma et al. (63) have also shown that some $CD8^+CD28^-$ T cells can regain CD28 expression. Some intriguing possibilities are suggested by the phenotypic analysis of BY55. While iIEL and the $CD8^+CD28^-$ peripheral blood subset clearly share some markers ($CD3^+CD8^+CD11b^+CD28^-$), others are different (CD101, CD103). The phenotypic similarities of iIEL and $CD8^+CD28^-$ peripheral blood T cells suggest that some iIEL may migrate into peripheral blood and vice versa. This has recently been documented in SIV infection (66). Thus, the $BY55^+CD8^+CD28^-$ T cell population may represent a terminally differentiated population with active cytolytic but limited proliferative capacity.

In cord blood, $BY55^+CD8^+$ T cells are not yet present, and all $BY55^+$ lymphocytes have an NK-like phenotype (18). The $CD8^+CD28^-$ T cell subset expands in both percentage and absolute number as humans age (70). It has been suggested that the $CD8^+CD28^-$ T cell subset may be in a state of replicative senescence based on shorter telomere lengths (71), and in HIV infection, telomeres in these cells are even shorter (72, 73). This leads to the idea that $BY55^+CD8^+$ T cells in HIV infection reflect the massive commitment of the immune system to antiviral cytolysis, but that these cells also represent a population with limited expansion possibilities, perhaps reflecting clonal exhaustion.

It is an interesting observation that BY55 is expressed only in the subpopulation of T and NK cells that are programmed for the final effector function, i.e., cytolysis. When considering all these observations, it seems that iIELs, cytolytic $BY55^+CD8^+$ peripheral blood T lymphocytes, and $BY55^+$ NK cells all share some common properties, viz., poor or null proliferative response (31, 55–57), activated lymphocyte phenotype (16, 18, 50, 74), and capacity for cytolytic activity (16, 17, 45, 46, 57). Another important characteristic shared by iIELs and $BY55^+$ T lymphocytes is their expansion in certain disease conditions. For example, $BY55^+CD8^+$ T lymphocytes showed a fourfold increase in HIV infection (18). The peripheral blood $CD8^+CD28^-$ T cell subset is expanded following bone marrow transplantation (75), during chemotherapy (76), and in hereditary hemochromatosis (77), perhaps because it may be extrathymically derived (52). Similarly, increased numbers of iIELs have been reported in diseases of mucosal delayed-type hypersensitivity, allograft rejection, graft-vs-host disease, parasite infections, enteral challenge after immunization, and celiac disease (48, 49).

The restricted expression of BY55 on cytolytic lymphocytes and its homology to KIR suggest a role in target recognition. BY55 would not be expected to deliver an inhibitory signal as it lacks an immunoreceptor tyrosine-based inhibition motif; however, GPI-anchored proteins can signal by associating with kinases (78). The molecular cloning of BY55 will facilitate additional studies on its function in cytolytic lymphocytes.

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