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Ly-6I, a New Member of the Murine Ly-6 Superfamily with a Distinct Pattern of Expression

David L. Pflugh, Stephen E. Maher, and Alfred L. M. Bothwell

A new member of the mouse Ly-6SF, designated Ly-6I, has been isolated as a gene homologous to a segment of the Ly-6C gene. A single allelic difference in the mature protein sequence was identified, which is similar to other Ly-6SF members. Ly-6I mRNA has been detected in a wide range of tissues and cell lines, and a rabbit polyclonal Ab has been used to determine that Ly-6I protein is present at a low constitutive level on cell lines from several different lineages. In contrast to Ly-6C and Ly-6A/E, the Ly-6I gene is only weakly responsive to IFNs. Expression in vivo is most abundant on bone marrow populations and is coexpressed with Ly-6C on granulocytes and macrophages. However, Ly-6I is also expressed on immature B cell populations that do not express Ly-6C. Expression on mature B cells in spleen is uniformly low. Similarly, Ly-6I is expressed on TCRlow/int, but not TCRhigh, thymocytes. Ly-6I is re-expressed on Ly-6Chigh T cells in the periphery. Thus, Ly-6I may be a useful marker to define maturation stages of both T and B lymphocytes as well as subsets of monocytes and granulocytes. The Journal of Immunology, 2000, 165: 313–321.

The Ly-6 superfamily of genes (Ly-6SF) was initially defined at the molecular level with the cloning of the Ly-6E1 cDNA (1). A large cluster of cross-hybridizing genes is located on murine chromosome 15 (2) and includes the known genes Ly-6A/E (3), Ly-6C (4, 5), Ly-6F and Ly-6G (6), and Ly-6H (7). Other related but nonhybridizing genes, such as Thb/Ly-6d (8) and TSA-1/Sca-2 (9), are also located near this cluster. The Ly-6SF complex exists in two allelic forms, with Ly-6.1 alleles generally being expressed at lower levels than their Ly-6.2 counterparts (10). Human homologues were first recognized with the identification of CD59, which has a well characterized role as an inhibitor of the complement membrane attack complex (11–13). Certainly, CD59 homologues have been characterized in rabbit (14), rat (15), pig (16), and mouse (17). At the present time human homologues for several mouse Ly-6SF members are known, including Thb (18), Ly-6H (19), and TSA-1/Sca-2/RIG-E (20, 21). In addition, the human urokinase plasminogen activator receptor (uPAR) consists of three Ly6SF domains (22, 23).

Given the large number of Ly6SF Ags, they may perform a wide variety of functions. Gene knockouts have only been analyzed for Ly-6A/E, which suggested that the Ag may have an inhibitory role in regulating cell activation (24). Other studies have suggested roles in cell adhesion in lymphocytes (25) and bone marrow stroma (26) and in T cell Ag-specific immune responses (27). Overexpression of Ly-6A/E can permit CD4+ T cells to be positively selected in the absence of MHC expression (28). The Ly-6A/E Ag was used to purify the long-term reconstituting hematopoietic stem cell (29). Other Ly-6SF members have been associated with specific cell types. The recently identified lynx1 is expressed in specific neural cell types and may regulate cholinergic function (30). Ly-6C may be a useful marker for memory CD8 T cells (31) and an adhesion molecule for endothelial cells (32) and splenic macrophage progenitor (33), while Ly-6G has commonly been used as the Gr-1 marker for granulocytes (6).

We have previously characterized the rearranged promoter in the Ly-6C gene in NOD mouse DNA that results in a 95% reduction of Ly-6C expression (34). However, as in the Ly-6A/E knockout mouse, this deficiency in Ly-6C has no apparent effect on the development of any lymphoid population. One possibility is that multiple Ly-6 proteins with similar functions are present on immature leukocytes. A region of the NOD Ly-6C promoter was identified where a retroposon integration occurred, and a 1-kb DNA fragment from that region showed evidence of hybridization to only two chromosomal genes, presumably Ly-6C plus an unknown. The chromosomal genes for these two genes have been isolated, and one is a new gene family member designated Ly-6I. Ly-6I is shown here to be expressed on a wide variety of cell and tissue types, many of which also express Ly-6A/E or Ly-6C.

Materials and Methods

Mice

C57BL/6 and NOD mice were bred and maintained in a pathogen-free environment at Yale University School of Medicine (New Haven, CT). Lymphocytes were isolated from animals at 4–6 wk of age.

Cell lines

The B cell lymphoma M12, the T cell lymphoma BW5147, the monocyte cell line J774, and the fibroblast-like cell line NIH-3T3 were cultured in DMEM containing 10% FCS with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The BALB/c plasmacytoma S194 was provided by Dr. Simon Carding (Department of Microbiology, University of Pennsylvania Medical Center, Philadelphia, PA) and grown in RPMI with 10% horse serum (Gemini, Calabasas, CA), 2 mM glutamine, 5 × 10−5 M 2 ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. The BALB/c keratinocyte cell line PAM-212, obtained from Dr. Robert Tigelaar (Yale Medical School), was cultured in IMDM with 10% FCS, 2 mM glutamine, 5 × 10−5 M 2 ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. To assess the induction of Ly-6I expression, cells were treated for 18 h with
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Isolation of phage genomic clone

The strain 129 D3 genomic DNA library from Stratagene (La Jolla, CA) was screened at high stringency using standard techniques (35). The probe was a 1-kb EcoRI-PstI DNA fragment derived from the Ly-6C gene promoter (Brosly described (34)). By restriction analysis, two clones were found to represent a single novel Ly-6 gene family member. Hybridization with an Ly-6c1 cDNA probe revealed the proposed coding region of the gene, which was contained within an ~7.5-kb EcoRI-SalI fragment and subcloned into pHBluescript. As this genomic DNA was 80% identical with Ly-6a/E and Ly-6c, the new gene has been termed Ly-6I (GenBank accession no. AF231406).

cDNA detection and cloning

Whole cell RNA was prepared from mouse bone marrow cells or subconfluent cultures of various cell lines with TRIzol reagent (Life Technologies, Grand Island, NY). Mouse tissue RNA from outbred Swiss mice was purchased from Ambion (Austin, TX). RT of 5 µg of RNA was accomplished with the ThermoScript RT-PCR system (Life Technologies) using an oligo(dT)20 primer. Ly-6I2 cDNA was isolated from both C57BL6 mouse bone marrow-derived macrophages and HeLa cells transfected with the EcoRI-SalI subclone using the forward primer 5’-CGCGAATTCTCGAGCTG and the reverse primer 5’-GTAATCAGATCTAATGGTGATGGTGATGATGGGA (forward) and 5’-GGGTGGGGACCATCACATCAG-3’ (reverse) and PstI are underlined) and the reverse primer 5’-CGCGAATTCTCGAGCTGACATCACATCA G3’-EcoRI and SalI are underlined). The two nucleotides in the reverse primer indicated in bold are point mutations introduced to generate the SalI site. The last nucleotide in the forward primer differs from the genomic sequence in Fig. 1 due to an error in the original sequence of the genomic DNA. The first 14 nucleotides of the forward primer and the first 12 nucleotides of the reverse primer are linker sequences used to add the EcoRI sites. A smaller fragment containing only the coding region of the mature protein was obtained with a second forward primer 5’-CGGGAAGTG-CAAGGTGTTACCAG-3’ (PstI is underlined). PstI was introduced by the point mutation shown in bold, which changed the second amino acid of the mature protein from glutamic acid to glutamine. This second fragment was used as previously described (16) to generate an MHC-tagged Ly-6I2 expressed in CHO cells.

Ly-6I1 cDNA was isolated in two alternately spliced forms (GenBank accession no. AF232024) from S194, PAM-212, and NOD bone marrow RNA with the forward primer 5’-CTCGAGCCAGTCTGAGAGGA3’ and the reverse primer 5’-GGGTGGGGACCATCACATCAG-3’. These primer recognition sequences correspond to the first exon to the first intron of the mRNA sequence. Optimal PCR conditions employed Platinum Taq High Fidelity polymerase (Life Technologies), Optimized Buffer B (Invitrogen, Carlsbad, CA), and a 60°C annealing temperature. PCR fragments were agarose gel purified with GFX columns (Amersham Pharmacia Biotech, Piscataway, NJ) and cloned into the vector pCR-Blunt II TOPO (Invitrogen). DNA sequences were determined using ThermoSequenase II dye terminators (Amersham Pharmacia Biotech) analyzed with a 373 DNA Sequencer (Applied Biosystems, Foster City, CA).

Rabbit polyclonal anti-Ly-6I

A soluble form of Ly-6I2 mouse was produced with the Drosophila Expression System (Invitrogen). Using the construct Ly-6I2 as template, a PCR product was generated with the primers 5’-CTTGTAGATCCTGGATGTACCGG-3’ (forward) and 5’-GATATACAGCTAAATGTTGTTGTTGTTGGGA GCTGCCCTCAGG-3’ (reverse; BglII sites are underlined) and cloned into the BglII site of the pMT/BiP/V5-His A vector. His6 was directly encoded by the reverse primer. The expression vector containing the Ly-6I-His6 chimera was cotransfected together with pCISYGRO into S2 Drosophila cell line transfected with calcium-phosphate, and lysates were produced and subjected to Ni-Sepharose. Isolates from pools of transfectants were generated. This construct uses the insect BIP protein to direct secretion of the recombinant protein into the culture medium. Expression of the chimeric protein was induced with 500 µM cupric sulfate for 4 days. Supernatants from individual pools were screened for Ly-6I expression by Western blot with an anti-His6 mAb. One pool was expanded for larger scale preparation of secreted Ly-6I, which was purified on a Ni-Sepharose column (Invitrogen). The highest purity of the recombinant protein was achieved by extensively washing the column with a 20 mM phosphate and 500 mM NaCl, pH 5.0, buffer followed by elution with 20 mM phosphate and 500 mM NaCl, pH 4.0. Buffer exchange and concentration of the eluted protein were performed with spin concentrators with a 5-kDa M, cut-off (Vivaspin, Westford, MA). Approximately 1 mg of recombinant protein could be isolated from 400 ml of supernatant. Harlan BioProducts for Science (Indianapolis, IN) used the purified protein to produce rabbit antisera recognizing Ly-6I. The polyclonal Ab was titrated by flow cytometry of the Ly-6Imyc tag CHO transfectant and bone marrow cells. A 1/250 dilution of the antisera was used to detect Ly-6I on cell lines, and a 1/100 dilution was used to stain normal tissues.

Abs and flow cytometry

Abs to CD4 (GK1.5), CD8 (53-6.72), Ly-6A/E (Sca-1), and Ly-6C (HK1.4) were purified from hybridoma supernatants with GammaBind G Sepharose (Amersham Pharmacia Biotech) and modified with N-hydroxysuccinimimidobiotin (Sigma, St. Louis, MO). FITC-conjugated avidin (Vector Laboratories, Burlingame, CA) and streptavidin-Cy-Chrome (PharMingen, San Diego, CA) were used as secondary reagents for single-color- or triple-color flow cytometry, respectively. FITC-conjugated F(ab)2 donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used to detect staining with the rabbit (Rb) polyclonal anti-Ly-6I antibody. Binding of the GammaBind-purified 9E10 mAb to the Myc tag was detected with FITC-conjugated F(ab)2 goat anti-mouse IgG (Fcy-specific, Jackson ImmunoResearch Laboratories). All secondary Abs were cross-species absorbed to assure specificity. mAbs against CD4, CD8, CD11b, CD19, Gr-1, TCRγ, TCRδ, TER-119, and NK1.1 were obtained from Pharmingen as FITC, PE, and/or biotin conjugates. Negative controls were normal rat IgG conjugated with PE (PharMingen), FITC, or biotin (Jackson ImmunoResearch Laboratories). Working dilutions of all Abs were determined in PBS with 0.1% sodium azide and 5% normal rat serum and 100 µg/ml mouse γ-globulin (Jackson ImmunoResearch Laboratories) to prevent nonspecific staining. Flow cytometric analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA) with CellQuest software. To examine monocytes and granulocytes in the spleen, cells were stained with PE-conjugated anti-Gr-1 or anti-CD11b, Rb preimmune or anti-Ly-6I serum plus FITC-conjugated donkey anti-Rb IgG and biotinylated Abs to CD4, CD8, and CD19 plus streptavidin-Cy-Chrome. All Cy-Chrome-positive cells were gated out, and only Cy-Chrome-negative cells were collected for analysis. Similarly, spleen cells stained for PE-conjugated anti-TCRβ were positively selected for analysis of Ly-6I vs Ly-6A/E and Ly-6C expression.

Results

Isolation of the chromosomal Ly-6I gene

A 1-kb EcoRI-PstI fragment derived from the Ly-6C gene was used to isolate phage clones from a D3 strain 129 DNA library. Consistent with previous Southern blot results (34) two classes of recombinant phage were isolated. One corresponded to the Ly-6C2 gene from 129 DNA, and the other was a new gene. Analysis of hybridization of phage DNA with the Ly-6E1 cDNA revealed a 7.5-kb EcoRI-SalI fragment that contained all the hybridizing DNA. This region and numerous PstI, SacI, and BglII subclones were isolated and subcloned into pBS. The DNA sequence was determined for 5 kb at the EcoRI end, which contained the entire coding sequence (Fig. 1).

Comparison with the cDNA sequence of Ly-6c DNA suggested the same exon/intron organization as we have previously characterized for the Ly-6C and Ly-6E genes (3, 4). The underlined DNA sequences represent the homologous exon boundaries. The 5’-flanking region shown contains 1161 bp. The region of the Ly-6C2 probe that was used to isolate the Ly-6I gene shows 86% identity with the Ly-6I DNA sequence (90-700 bp shown in Fig. 1). The region of the promoter immediately 5’ to the first exon between Ly-6I and Ly-6C is especially homologous, while exon 3 is 91% identical with the same region of Ly-6A/E. The entire gene is 80% identical with both Ly-6C1 and Ly-6A,2 genes.

To functionally characterize the Ly-6I gene, the EcoRI-SalI subclone was cotransfected with pSV2neo into HeLa cells. Individual clones from isolated and, Northern blot analysis of total RNA revealed a 1-kb mRNA species (data not shown) similar in size to that observed in murine cells for the expressed Ly-6A/E and
Ly-6C mRNAs (36). Thus, this subclone contained a functional promoter and expressed an Ly-6-like mRNA.

The exons of the Ly-6I gene were defined by homology with other Ly-6 genes. From these putative exons, an amino acid sequence was derived (Fig. 1). The protein potentially expressed by the Ly-6I gene appeared similar in size to other known Ly-6 proteins, and Ly-6I contained the 10 conserved cysteine residues characteristic of Ly-6SF. Direct comparison of the Ly-6I amino acid sequence with other murine Ly-6 molecules demonstrated that the cysteine residues also occur at relatively conserved intervals as well (Fig. 2). Calculating the percent similarity between the Ly-6 proteins, which measures both identity and conservative amino acid changes, Ly-6I is 70% homologous to the cross-hybridizing Ly-6SF members Ly-6A/E, Ly-6C, Ly-6F, and Ly-6G, but is much more distantly related to five other known murine Ly-6 proteins. Most of the homology occurs in the extracellular signal sequence (aa 22 to 121) and the glycosyl phosphatidylinositol (GPI) attachment signal region (aa 80 –108). Highly conserved among all the Ly-6 proteins is the region immediately N-terminal to the GPI sequence. The similarity of exon 3 between Ly-6I and Ly-6A/E is also readily apparent, with 29 of the first 36 amino acids being identical.

FIGURE 1. Nucleotide sequence of the Ly-6I gene. The four exons are underlined and defined by comparison of the cDNA with the genomic DNA sequence, except for the extreme 5’ and 3’ termini, which are defined by homology with the Ly-6A/E gene. The translated amino acid sequence in single-letter code is shown for exons 2–4. Restriction endonuclease sites are noted above the DNA sequence.
Characterization of the cDNA

The RNA from several cell lines and tissues was analyzed by RT-PCR, because Northern blotting would not distinguish cross-hybridizing Ly-6SF members. Most mouse tissues contained Ly-6I mRNA, with the strongest signal in liver, thymus, spleen, and kidney (Fig. 3A). Bone marrow also typically contained an intense signal comparable to that of spleen and thymus (not shown). Reproductive tissue (testes and ovary) contained the least amount of the expected PCR product of 498 bp. Expression of two distinct size classes was observed in cell lines (Fig. 3B). The product obtained from several lines, such as M12, S194, BW5147, and NIH-3T3, corresponded to that expected from an accurately spliced product (represented by S194). By contrast, P815 (not shown) and PAM-212 cells expressed a product; ~200 bp larger. J774 cells also showed the larger PCR product in addition to the expected fragment, but after IFN-γ activation only the smaller band was seen.

FIGURE 2. Comparison of Ly-6I with other mouse Ly-6 proteins. To limit the sequences for comparison, only the 0.2 alleles are given where known; otherwise, the 0.1 allele is shown. The Ly-6d and Ly-6H proteins do not differ between the two alleles. The Ly-6H sequence shown begins with the second methionine residue. Dashes indicate identity, while periods denote gaps. The 10 conserved cysteine residues are marked with asterisks. The percent similarity of each protein with Ly-6I.2 is given at the end of each sequence. Ly-6E.1 has an alanine at residue 80 as one of two allelic differences from Ly-6A.2 and thus has a 74% similarity to Ly-6I.

Characterization of the cDNA

The RNA from several cell lines and tissues was analyzed by RT-PCR, because Northern blotting would not distinguish cross-hybridizing Ly-6SF members. Most mouse tissues contained Ly-6I mRNA, with the strongest signal in liver, thymus, spleen, and kidney (Fig. 3A). Bone marrow also typically contained an intense signal comparable to that of spleen and thymus (not shown). Reproductive tissue (testes and ovary) contained the least amount of the expected PCR product of 498 bp. Expression of two distinct size classes was observed in cell lines (Fig. 3B). The product obtained from several lines, such as M12, S194, BW5147, and NIH-3T3, corresponded to that expected from an accurately spliced product (represented by S194). By contrast, P815 (not shown) and PAM-212 cells expressed a product ~200 bp larger. J774 cells also showed the larger PCR product in addition to the expected fragment, but after IFN-γ activation only the smaller band was seen. However, these primers do show that the mRNA is expressed in many mouse cell lines from a variety of lineages. The primers used to detect the cDNA were specific for Ly-6I, as restriction analysis of RT-PCR products from 13 different cell lines and tissues and sequencing of individual cloned revealed no evidence of the primers cross-reacting with any other Ly-6 gene.

To determine whether allelic differences occurred within the Ly-6I gene, PCR products obtained from S194 and PAM-212 cells were cloned and sequenced. The DNA sequences of these PCR products are summarized in Fig. 4, with the differences between the Ly-6I.1 and Ly-6I.2 products indicated. The S194 cDNA was actually from a differentially spliced mRNA (double arrows in Fig. 4), which is 46 bp shorter than the cDNA originally isolated from bone marrow and the HeLa transfectant. The larger PCR product from PAM-212 cells was a normal mRNA, except that the 212-bp intron 1 was still present. In the 710-bp sequence shown (from PAM-212 cells) there are five base pair differences detected between Ly-6I.1 and Ly-6I.2: 1 bp in the first exon of the normal mRNA form, 1 bp in the intron, 2 bp in the leader sequence, and 1 bp in exon 4 encoding the mature protein. One change in the leader alters the sixth amino acid residue from glutamic acid to alanine, and the point mutation in codon 42 results in a lysine in the 0.2 allele and an arginine in the 0.1 allele. This type of allelic difference is also found in the Ly-6C alleles (residue 59) (4, 5) and in the mouse IgM alleles (37). Similarly, an amino acid difference at residue 37 is responsible for the allelic differences in Ly-6E.1 (1) vs Ly-6A.2 (38). A potential site for N-linked glycosylation occurs at position 69, which marks a difference among Ly-6I, Ly-6A/E, and Ly-6C. Of the known murine Ly-6SF molecules, only Ly-6G, Ly-6H, and CD59 also have possible N-glycosylation sites.

Detection of Ly-6I protein

Based on the gene sequences, primers were synthesized and used to isolate a cDNA by RT-PCR that would encode the Ly-6I protein. To test whether this cDNA could indeed result in a protein expressed on the cell surface, a fusion protein was created that contained an epitope tag. The human CD59 leader sequence plus the 11-residue Myc tag was fused to codon 1 of the Ly-6I.2 sequence. We have previously used this strategy to tag the porcine CD59 (16). The chimeric cDNA was subcloned into the pFRSV-SR expression vector (39, 40) and transfected into CHO cells whose expression levels were subsequently amplified using methotrexate. Amplification was necessary because transfected Ly-6
genes are generally difficult to express at high levels, and some transfectants lose Ly-6 expression after a period of time in culture. FACS analysis of the transfected CHO cell line showed high levels of Ly-6I detected with the 9E10 anti-Myc epitope tag mAb (Fig. 5A).

To directly examine the expression of Ly-6I in cell lines and normal cells and tissues, a polyclonal Ab was developed in rabbits. To obtain sufficient amounts of pure protein, a soluble Ly-6I fusion protein was created with a His6 carboxyl-terminal tag replacing the final 20 aa of the GPI anchor sequence. The recombinant protein was expressed in Drosophila S2 cells and was directed for secretion by the insect BiP leader sequence. The secreted recombinant protein was purified on a nickel column by virtue of the His6 tag and used to immunize rabbits. The resultant antiserum readily detected the Myc-tagged Ly-6I that had been expressed at high levels on CHO cells with a similar pattern of staining as the 9E10 mAb (Fig. 5A). The different intensity of staining obtained with the two Abs may be due to the Rb polyclonal Ab recognizing multiple epitopes on Ly-6I or to some proteolytic removal of the Myc tag. The different background levels on normal CHO cells were the result of the different secondary Abs used.

This antisera has also been used to assess the expression of several murine cell lines (Fig. 5B), and the results are generally consistent with the RT-PCR data. This Ag is expressed at relatively low levels on many different cell lines, three of which are shown in Fig. 5. In S194 cells there was no apparent increase in Ly-6I expression when the cells were treated with either IFN-α/β or IFN-γ. However, as expected from the RT-PCR (Fig. 3), there was a slight increase in the level of expression on the J774 macrophage cell line, from a mean fluorescence index (MFI) of 49.0 on untreated cells to a MFI of 75.6 after IFN-γ treatment. NIH-3T3 fibroblast cells also showed a slight increase in Ly-6I on the cell surface. In contrast, much greater increases in the expression of Ly-6A/E were seen following IFN treatment of these two cell lines. Ly-6I expression on these cell lines indicates that the alternatively spliced or even unspliced forms detected by RT-PCR can result in protein expression. Both PAM-212 and P815, which exclusively express the unspliced form, are Ly-6I positive by flow cytometry (data not shown). Thus, the lack of splicing in this 5’ untranslated region has no apparent negative effects on protein translation.

Expression of Ly-6I in granulocytes and monocytes

Bone marrow was examined as a source of Ly-6I expression, because analysis of tissues by RT-PCR indicated the presence of Ly-6I mRNA, and the Ly-6I promoter showed homology to that of Ly-6C, which is expressed at the highest levels in bone marrow. The data in Fig. 6 show analysis of C57BL/6 and NOD bone marrow cell populations using the Rb antiserum. The comparison with NOD was chosen for two reasons: C57BL/6 is an 0.2 allele, while NOD is an 0.1 Ly-6 allele, and the expression of Ly-6C in NOD is reduced compared with that in wild-type C57BL/6 bone marrow. This is very apparent when the expression of Gr-1 vs Ly-6C is examined (Fig. 6, A and E). The percentage of cells expressing high levels of Ly-6C is significantly reduced in NOD bone marrow. When expression of Ly-6I on Gr-1 cells is examined, there are significant populations of cells that stain with both Abs (Fig. 6, C and G). The staining pattern is similar between C57BL/6 and NOD cells, although the NOD cells do express lower levels of both Ly-6I and Ly-6C compared with the 0.2 alleles of C57BL/6. As expected, because most Gr-1+ bone marrow cells also express CD11b, the same pattern is seen when cells are costained for Ly-6I.

FIGURE 4. Comparison between Ly-6I.1 and Ly6I.2 alleles. The Ly-6I.1 sequence represented is the long RT-PCR product derived from PAM-212 cells. The nucleotide sequence is given in lower case, while the single-letter amino acid abbreviations are given in upper case. The differences with Ly-6I.2 are shown below each site. The single arrows indicate the splice sites observed in the HeLa cell transfected with the genomic clone, and double arrows indicate the shorter spliced product from S194 RNA. The single-underlined base pair indicates the primers used to isolate the cDNA by RT-PCR. The double-underlined nucleotides mark the primers used to construct the soluble His6 fusion protein, and the forward primer was in the same region as that used to generate the Myc-tagged version of Ly-6I. The underlined amino acids indicate the potential N-linked glycosylation site.
and CD11b. These data show that expression of Ly-6I is very high on some cell populations, but is not dependent on Ly-6C. The expression of Ly-6I slightly differs from that of Ly-6C on mature granulocytes and monocytes. Ly-6C defines two populations of granulocytes in the spleen: Gr-1<sup>high</sup> Ly-6C<sup>int</sup> cells and Gr-1<sup>int</sup> Ly-6C<sup>high</sup> cells (Fig. 6I). However, staining for Ly-6I resolves the Gr-1<sup>high</sup> cells into two groups depending on the intensity of Ly-6I expression (Fig. 6K). Costaining for Ly-6I and CD11b on non-T/non-B spleen cells results in an extremely complex pattern (Fig. 6L). Some of the populations correspond to Gr-1<sup>high</sup> cells, while others may represent heterogeneity of monocytes. Little or no Ly-6I can be detected on NK1.1<sup>1</sup> cells or TCR<sup>gd</sup>1 T cells in the spleen (data not shown), so these cell types at least partially comprise the double-negative population.

Expression of Ly-6I by lymphocytes

Other lineages in the bone marrow were examined for Ly-6I expression. Although TER-119<sup>1</sup> erythroid cells were Ly-6I<sup>2</sup> (data not shown), CD19<sup>1</sup> B cell populations showed low levels of Ly-6I expression (Fig. 7C). In contrast, most of the same cells expressed little Ly-A/E (Fig. 7A) or Ly-6C (data not shown). Analysis of B cells from spleen indicates that the CD19<sup>+</sup> cells have even lower levels of Ly-6I expression (Fig. 7F). Again, this contrasts with Ly-6A/E, which was present on a majority of splenic B cells in this experiment (Fig. 7D).

T cells in the thymus and spleen were also tested for Ly-6I expression (Fig. 8). Most thymocytes were Ly-6I<sup>1</sup>, with the highest levels of expression on TCR<sup>low/int</sup> cells, while the TCR<sup>high</sup> cells were mainly Ly-6I<sup>1</sup> (Fig. 8B). This correlates with B cell expression of Ly-6I, where expression levels also appear to decline as the lymphocytes mature. In the spleen, a small population of T cells were Ly-6I<sup>1</sup> (Fig. 8D). This population was approximately the same size (6.2 vs 5.6% of total splenocytes) as the Ly-6C<sup>high</sup> T cells (Fig. 8F). When T cells are costained for Ly-6I and either Ly-6A/E or Ly-6C, the expression of Ly-6I correlates only with Ly-6C (Fig. 8H). These Ly-6I<sup>1</sup>/Ly-6C<sup>high</sup> T cells are presumably activated T cells. The functional significance of the Ly-6A/E<sup>high</sup>Ly-6C<sup>low</sup> and the Ly-6C<sup>high</sup>Ly-6I<sup>low</sup> populations is unknown, but each group represents ~3% of total splenic T cells. Thus, Ly-6I appears to be expressed on lymphocyte precursors, and although mature cells are Ly-6I<sup>low</sup>, Ly-6I may be re-expressed following lymphocyte activation.

Discussion

A novel murine gene has been identified as a member of the Ly-6SF and has been designated Ly-6I. The entire coding sequence of the Ly-6I gene has been cloned and sequenced. Ly-6I has an intron/exon structure similar to those of other known Ly-6SF genes, and the predicted amino acid sequence reveals 10 cysteine residues at certain intervals that are a defining characteristic of this gene family. Ly-6I was isolated by homology to the Ly-6C gene and is ~80% identical at the molecular level and 70% similar at the protein level to a subset of the Ly-6SF that also includes Ly-6A/E, Ly-6C, Ly-6F, and Ly-6G. Similar to Ly-6A/E and Ly-6C, a single allelic difference was identified in Ly-6I proteins from Ly-6.1 and Ly-6.2 mouse strains. A polyclonal Ab generated against a soluble form of Ly-6I was able to detect protein expression on a variety of cell lines. Flow cytometric analysis of Ly-6I expression on myc-Ly-6I transfected or normal CHO cells (A) and S194, J774 cells, and NIH-3T3 cells (B) with or without IFN treatment. Staining with Rb anti-Ly-6I (thin line) was compared with that with preimmune serum (thick line) from the same animal. The MFI for Ly-6I is shown in each histogram in B. The Myc-tagged Ly-6I (A) was also detected with the 9E10 mAb (dotted line). IFN induction of Ly-6A/E expression (B) was detected with the Sca-1 mAb (dotted line).
murine cell lines and on many freshly isolated hemopoietic cells. Although it was raised against one member of highly homologous family of proteins, the polyclonal Ab appears to recognize only Ly-6I. Direct binding studies have been performed with Ly-6A/E and Ly-6C transfectants in addition to an ELISA using soluble forms of Ly-6A/E, Ly-6C, and Ly-6I, and no cross-reaction of the polyclonal Ab was observed (data not shown). Thus, Ly-6I is a functional member of the Ly-6SF with a unique pattern of expression.

Ly-6I was isolated through the homology of its promoter with a similar region of the Ly-6C gene. Therefore, it was probable that Ly-6I would be found on some of the same cell types as Ly-6C, which is most highly expressed in the bone marrow. Indeed, staining for Ly-6I and Ly-6C on immature granulocytes and monocytes in the bone marrow results in very similar patterns (Fig. 6). However, expression of Ly-6I is not altered in the NOD mouse, which is deficient in Ly-6C, so the polyclonal anti-Ly-6I Ab was, in fact, recognizing a protein other than Ly-6C. Ly-6I was also expressed by mature T cells with high levels of Ly-6C. However, T cells that express lower amounts of Ly-6C are Ly-6I- as are a very small subset of Ly-6C high T cells (Fig. 8). No Ly-6I has detected on NK1.1+ cells, again in contrast to Ly-6C. Although expression of

FIGURE 6. Expression of Ly-6I on Gr-1- and CD11b-positive cells in bone marrow. Bone marrow cells were isolated from C57BL/6 (A–D) and NOD (E–H) mice and costained for Gr-1 or CD11b vs Ly-6C (A and E) and Ly-6I (C, D, G, and H). Staining with Rb preimmune serum (B and F) is shown as a negative control. A similar analysis was performed on CD4+CD8−CD19− cells (I–L) from C57BL/6 spleen.

FIGURE 7. Expression of Ly-6I on CD19+ cells. Bone marrow cells (A–C) and splenocytes (D–F) from C57BL/6 mice were costained with anti-CD19 and Sca-1 (A and D) or Rb anti-Ly-6I (C and F). Staining with Rb preimmune serum (B and E) is shown as a negative control.
Ly-6I is similar to that of Ly-6C, this new gene is not expressed by all Ly-6C⁺ cells.

Expression of Ly-6I also partially overlaps that of other Ly-6SF members (10). Some peripheral T cells express both Ly-6I and Ly-6A/E, although most T cells express only one of these molecules. Ly-6I is also expressed on TCRlow/int thymocytes, which do not express Ly-6A/E. The majority of thymocytes are Ly-6I⁺ and also express Thb/Ly-6d and Sca-2/TSA-1, but splenic B cells express very weak levels of Ly-6I, in contrast to Ly-6A/E and Thb/ Ly-6d. In addition, Ly-6I expression is not limited to myeloid cells, as is Ly-6G (Gr-1). Many cell types express multiple Ly-6SF molecules, yet each family member, including Ly-6I, has a distinct pattern of expression.

The Ly-6I gene is surprisingly unresponsive to IFN. Expression of both Ly-6A/E and Ly-6C is up-regulated by IFN-α/β and IFN-γ (36). Only the J774 macrophage cell line demonstrated any notable increase in Ly-6I surface expression following IFN treatment (Fig. 5). Induction of Ly-6I with IFN may be limited to only a few cell lineages. In much the same manner, Ly-6C expression is up-regulated on fewer cell types by IFN than is Ly-6A/E. Elements of the Ly-A/E gene required for IFN responsiveness have been identified (41, 42), but the corresponding regions of the Ly-6I gene are 5’ of the fragment sequenced to date (Fig. 1). Further characterization of the Ly-6I promoter will be necessary to determine whether any of the IFN-responsive elements are absent or altered in the Ly-6I gene.

Ly-6I expression may be able to distinguish populations of lymphoid cells. Staining for Ly-6I separates Gr-1⁺ and CD11b⁺ splenocytes into several different subpopulations (Fig. 6), which cannot be delineated with Ly-6C. At least three different Gr-1⁺Ly-6I⁺ cell types were identified, whereas Ly-6C expression separates Gr-1⁺ cells into only two fractions. The CD11b⁺Ly-6I⁺ may include both the granulocyte subfractions as well as monocytes at different stages of activation. For example, Ly-6I expression may be up-regulated by IFN in normal monocytes as in the J774 cell line. Other populations that require further study are the Ly-6I⁺Ly-6A/E⁺Ly-6C⁺ T cells (Fig. 8, H and G) and the Ly-6I⁺Gr-1⁻CD11b⁻ bone marrow cells (Fig. 6, C and D). The Ly-6I⁺Gr-1⁻ T cells probably represent activated lymphocytes, but the bone marrow cells do not correspond to any lineage tested (data not shown).

Ly-6I may have some function in the differentiation of various hemopoietic lineages. High levels of Ly-6I are present on immature granulocytes and monocytes in the bone marrow. Lower amounts of Ly-6I are detectable on immature lymphocytes of both the T and B cell lineages. Ly-6I expression declines as the lymphocytes mature, as best seen by TCR vs Ly-6I expression in the thymus (Fig. 8B). Thus, Ly-6I could function in a common pathway for T and B lymphocyte development. Ly-6I also appears to be re-expressed by activated T cells in the periphery, making Ly-6I especially useful in characterizing different stages of T lymphocyte development and activation.

The function of most Ly-6SF members, with the exception of CD59 and uPAR, is still unknown. Studies of Ly-6A/E knockout mice (24) and disruption of the Ly-6C gene in NOD mice have revealed no function for these molecules in hemopoiesis. One explanation for these results is that some of the Ly-6SF members are functionally redundant, so that the loss of one gene is not sufficient to affect lymphoid cell development. Indeed, we have shown that immature granulocytes, monocytes, T lymphocytes, and B lymphocytes all express Ly-6I. Ly-6I is highly homologous to the Ly-6A/E, Ly-6C, and Ly-6G/Gr-1 proteins. Although most of the identity among these genes lies outside the coding region of the mature protein, all Ly-6SF members are expected to assume very similar three-dimensional structures based on the 10 highly conserved cysteine residues (30). Ly-6A/E and Ly-6C have been proposed to act as adhesion molecules by binding cell surface ligands (25, 32, 33). The conserved structure of the Ly-6SF may allow more than one family member to associate with a given ligand molecule. Alternatively, redundant Ly-6SF proteins may act by influencing the activity of other adhesion molecules, as in the case of uPAR associating with β₂ integrins (43). The broad tissue distribution of Ly-6I may be an indication that either it can interact with many different molecules or its target molecule is also widely expressed.

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References


40. Immuno-