LYSP100-Associated Nuclear Domains (LANDs): Description of a New Class of Subnuclear Structures and Their Relationship to PML Nuclear Bodies

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The PML gene is fused to the retinoic acid receptor α (RARα) gene in t(15;17) acute promyelocytic leukemia (APL), creating a PML-RARα fusion oncoprotein. The PML gene product has been localized to subnuclear dot-like structures variously termed PODs, ND10, Kr bodies, or PML nuclear bodies (PML NBs). The present study describes the cloning of a lymphoid-restricted gene, LYSPlOO, that is homologous to another protein that localizes to PML NBs, SP100. In addition to SP100 homology regions, one LYSPlOO cDNA isoform contains a bromodomain and a PHD/ITC domain, which are present in a variety of transcriptional regulatory proteins. By immunofluorescence, LYSPlOO was localized to nuclear dots that were surprisingly largely nonoverlapping with PML NBs. However, a minority of LYSPlOO nuclear dots exactly colocalized with PML and SP100. We term the LYSPlOO structures “LANDs,” for LYSPlOO-associated nuclear domains. Although LYSPlOO is expressed only in lymphoid cells, LANDs could be visualized in HeLa cells by transfection of a LYSPlOO cDNA. Immunelectron microscopy revealed LANDs to be globular, electron-dense structures morphologically distinct from the annular structures characteristic of PML NBs. LANDs were most often found in the nucleoplasm, but were also found at the nuclear membrane and in the cytoplasm, suggesting that these structures may traffic between the cytoplasm and the nucleus. By double-immunogold labeling of PML and LYSPlOO, some LANDs were shown to contain both PML and LYSPlOO. Thus, PML is localized to a second subnuclear domain that is morphologically and biochemically distinct from PML NBs.

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The PML protein localizes to this domain. Interest in the PML gene derives from its involvement in the characteristic t(15;17) translocation of acute promyelocytic leukemia (APL). Interest in the PML gene derives from its involvement in the characteristic t(15;17) translocation of acute promyelocytic leukemia (APL). By immunofluorescence, the wild-type PML protein localizes to five to 15 dot-like or ring-like domains in the nucleus. Using immuno-EM, PML was detected in annular or dot-like, electron-dense structures in the nucleoplasm, 0.3 to 1 μm in diameter. PML preferentially localized over the electron-dense outer ring of these structures. This PML-containing structure is morphologically similar to a subclass of NBs previously defined by EM (reviewed in Brasch and Ochs15), and thus we will term this structure a PML NB in the present study. Although the normal function of PML NBs has remained elusive, a clue to their importance comes from the observation that DNA viruses encode proteins that localize to PML NBs transiently and subsequently disrupt the structures.

The t(15;17) translocation of APL results in the expression of a fusion oncoprotein in which PML is fused to the retinoic acid receptor α (RARα). In APL cells, the characteristic PML NB structures are absent and, instead, PML is localized to a large number of much smaller nuclear dots. APL patients treated with retinoic acid undergo a clinical remission resulting from the differentiation of leukemic cells into mature granulocytes and a concomitant decrease in proliferation (see Grignani et al20 for review). Interestingly, treatment of APL cells with retinoic acid causes reformation of the normal PML NB structures, leading to speculation that disruption of these subnuclear domains is integral to malignant transformation by PML RARα. Wild-type PML can suppress the growth and malignant phenotype of transformed cells,21,25 again suggesting that interference with PML function might contribute to the pathogenesis of APL.
calizes with LYSP100 in some LANDs, thus defining LANDs as a new PML-containing subnuclear structure.

MATERIALS AND METHODS

Cloning of LYSP100. Polymerase chain reaction (PCR)-amplifiable cDNA enriched for lymphoid-restricted genes was obtained by subtractive hybridization of cDNA from the Burkitt's lymphoma line, Raji, with mRNA from the erythroleukemia line, K562. This amplified cDNA was used to construct a subtracted cDNA library as previously described. Twelve hundred ninety clones from the resultant subtracted library were sequenced by automated DNA sequencing and evaluated for homology using the Basic Local Alignment Search Tool (BLAST) algorithm. A partial cDNA clone, AD2511, was found to have high homology with the amino-terminal region of SP100. The AD2511 partial LYSP100 cDNA clone was used to screen a large-insert Lambda-ZAP (Stratagene La Jolla, CA) cDNA library prepared from the Raji cell line. cDNA library screening and other DNA manipulations were performed using standard techniques. DNA sequencing was performed using the Applied Biosystems (ABI; Foster City, CA) 373 Sequencer and PRISM (ABI) sequencingmixing sequences. The sequences of LYSP100 cDNA isoforms, LYSP100-A and LYSP100-B (Fig 2A), have been submitted to Genbank and assigned accession numbers U36499 and U36500, respectively.

Cloning of alternate SP100 isoforms. A PstI-XhoI fragment derived from LYSP100-A, which has sequences identical to the LYSP100-B human nuclear phosphoprotein (HNPP)-like domain, was used to screen a HeLa cDNA library (Stratagene). A 2.7-kb cDNA clone obtained from this screen, termed HFPX, was sequenced and found to contain a short open reading frame (49 amino acids) with very high homology to the carboxy-terminus of LYSP100-B. The region of HFPX with the highest homology to LYSP100-B was used to design the following nested antisense PCR primers: (1) outer primer, 5'-ATATCCGAATTCCCTTCACCGACCCAATCAGCAGGTCAC-3', and (2) inner primer, S'-TGGAAGTAACTCCTGGCCTCCAACTAAGTCTT-3'. These primers were used for reverse transcriptase-PCR using Raji mRNA and the following oligonucleotides: (1) sense, 5'-AAAAAAAAGGATCCAGGTTGAGTTGACAGAACACC-3', and (2) antisense, 5'-AAAAAAAGGATCCAGGTTGAGTTGACAGAACACC-3'. For SP100, DNA corresponding to amino acids 333 to 480 were obtained by reverse transcriptase-PCR using Raji mRNA and the following oligonucleotides: (1) sense, 5'-AAAAAAAGGATCCAGGTTGAGTTGACAGAACACC-3', and (2) antisense, 5'-AAAAAAAGGATCCAGGTTGAGTTGACAGAACACC-3'. PCR products were digested with BamHI plus EcoRI before cloning. An internal BamHI site in the SP100 gene (at amino acid 333) was used for glutathione-S-transferase (GST) cloning. GST alone, GST-SP100, and GST-LYSP100-A proteins were purified by standard procedures. Female New Zealand white rabbits were immunized with a primary boost of 500 µg soluble GST-fusion protein, followed by an initial boost with 200 µg protein 4 weeks later plus subsequent boosts every 2 weeks also with 200 µg protein. Blood samples were taken 12 days after every boost. GST alone and GST-fusion proteins were coupled to Affigel (BioRad, Richmond, CA) according to the manufacturer's specifications and used for affinity purification of LYSP100 and SP100 antibodies. Briefly, antisera was diluted 1:5 in 10 mmol/L Tris (pH 7.5) buffer and passed several times over a GST-alone Affigel column to remove anti-GST specificity. Diluted antisera was next passed over the respective GST-fusion protein column several times and washed extensively with Tris buffer. Antibodies bound to the column were eluted with 100 mmol/L glycine (pH 2.5) and then neutralized with 2 mol/L Tris (pH 7.5). Polyclonal mouse anti-SP100 antisera was generated against a maltose-binding protein–SP100-fusion protein as previously described.

Immunofluorescent cell staining and confocal microscopy. The Epstein-Barr virus–transformed human B lymphoblastoid cell line, MBB1, was obtained from Dr D. Allman (National Cancer Institute). Magnetic bead–purified (Dynal Corp, Lake Success, NY) CD19+ peripheral blood B lymphocytes were obtained from a tissue culture supernatant. Mouse anti-CD19 (Clone 4B-4A; PharMingen, San Diego, CA) was used for immunostaining of B cells. Nuclei were prepared according to the method of Xie et al. and whole-cell lysates were prepared according to the method used by Sambrook et al. Proteins were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel and then blotted onto nitrocellulose. After blocking with 2.5% nonfat dry milk solution, the filter was incubated with anti-LYSP100 antibodies at 1 µg/mL. Bound antibody was detected with the ECL system (Amer sham). Antibodies to LYSP100 and SP100. LYSP100 and SP100 were expressed in bacteria using the pGEX2tk vector (Pharmacia, Piscataway, NJ). For LYSP100, DNA corresponding to amino acids 306 to 412 of the A form was amplified by PCR from the LYSP100-A cDNA using the following oligonucleotides: (1) sense, 5'-AAAAAAAGGATCCAGGTTGAGTTGACAGAACACC-3', and (2) antisense, 5'-AAAAAAAGGATCCAGGTTGAGTTGACAGAACACC-3'. For SP100, DNA corresponding to amino acids 333 to 480 were obtained by reverse transcriptase-PCR using Raji mRNA and the following oligonucleotides: (1) sense, 5'-AAAAAAAGGATCCAGGTTGAGTTGACAGAACACC-3', and (2) antisense, 5'-AAAAAAAGGATCCAGGTTGAGTTGACAGAACACC-3'. PCR products were digested with BamHI plus EcoRI before cloning.
BioRad Comos software. Confocal images were merged using Adobe Photoshop 3.0 (Adobe Corp, Mountain View, CA).

Transfection of HeLa cells. A mammalian expression vector for LYSPl00-B was created by cloning LYSPl00-B cDNA into the vector pCGN\textsuperscript{37} as follows. LYSPl00-B cDNA was amplified by PCR using the following primers: S'TIITITGGTACCTGGCCC-AGCAAGGGCCAGCAGG3' and 5'TTfTTTGGATCCTATTTT-AGGGTGCCATTTGCTGAAT3'. The PCR product was digested with KpnI and BamH\textit{I} and ligated into the same sites in pCGN. For transfection, HeLa cells were plated at 25\% confluency on glass cover slips in the wells of 24-well plate and grown in Dulbecco's modified essential medium supplemented with 10\% fetal calf serum. Cells were transfected with the CaPO\textsubscript{4} procedure\textsuperscript{35} using 1 μg pCGN-\textit{LYSPl00-B} DNA per well. After addition of DNA, cells were incubated for 16 hours at 37°C and then placed in fresh media. Cells were grown on the cover slips for an additional 24 hours before staining with anti-LYSPl00 antibodies, as already described.

\textbf{Immunno-EM.} MBB1 cell cultures were fixed for 1 hour at 4°C with 4\% formaldehyde in 0.1 mol/L phosphate buffer (pH 7.3). During fixation, the cells were scraped from the plastic substratum and centrifuged. Pellets of fixed material were dehydrated in increasing concentrations of methanol and embedded in Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, Germany) at low temperature as previously described.\textsuperscript{38} Polymerization was performed for 5 days at −30°C under long-wavelength UV light. Formvar carbon-coated copper grids (mesh 200) bearing ultrathin sections of Lowicryl-embedded material were stained for immunogold detection by first incubating in a bovine serum albumin solution for 30 minutes to suppress nonspecific binding. Grids were then floated on 10-μL drops of an LYSPl00 antibody solution diluted to 10 μg/mL in PBS for 2 hours at 25°C. After a 15-minute wash in PBS, the grids were floated for 30 minutes on a 10-μL drop of a 1:25 dilution of goat anti-rabbit IgG conjugated to gold particles 10 nm in diameter (Biocell Research Laboratories, Cardiff, UK). For double staining, uncoated grids (mesh 300) were first stained for LYSPl00 on one side as already described, and then the opposite sides of the grids were stained similarly for PML using 15-nm gold particle-conjugated anti-rabbit IgG. As a specificity control for the immunogold labeling, all steps were performed normally but the primary antibody was omitted.

\textbf{RESULTS}

\textbf{LYSPl00 gene characterization.} In an effort to identify novel lymphoid-restricted transcription factors, we combined subtractive cDNA methodology with random automated DNA sequencing. A subtracted cDNA library was prepared that was enriched for genes expressed in the Burkitt's lymphoma cell line, Raji, and not in the erythroleukemia cell line, K562.\textsuperscript{32} One subtracted cDNA clone was chosen for study by virtue of its homology with an autoantigen, SP100\textsuperscript{26,27} that had been previously localized to PML NB subnuclear domains.\textsuperscript{2} Northern blot analysis showed that this gene was expressed as three major mRNA transcripts, 0.9, 1.6, and 3.0 kb in length, that were detected in all three lymphoid tissues examined (spleen, thymus, and tonsil) but not in three nonlymphoid tissues (Fig 1). LYSPl00 mRNA was expressed in all mature B-lymphocyte and plasma cell lines tested (Fig 1, and data not shown), but it was only variably expressed in T-lymphocyte lines and was not detectable in several nonlymphoid cell lines (Fig 1). Expression of the gene is thus lymphoid-restricted both in human tissues and in cell lines. Consequently, we named this gene LYSPl00, for lymphoid-restricted homologue of SP100. By contrast, SP100 expression is widespread\textsuperscript{22,29,27} (and A.L. Dent, unpublished results, January 1994).

To isolate full-length LYSPl00 cDNAs, a Raji cDNA library was screened with the partial LYSPl00 cDNA, and two cDNA clones, both 3.0 kb in length, were chosen for further analysis (Fig 2A). The sequences of LYSPl00-A and LYSPl00-B cDNAs\textsuperscript{2} have identical 5' and 3' sequences but differ by two short insertions in the middle of the LYSPl00-B cDNA, which probably result from alternative splicing.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{LYSPl00_fig1}
\caption{Expression of LYSPl00 mRNA in human lymphoid cell lines and tissues. A Northern blot was prepared using 2 μg poly(A)\textsuperscript{+} mRNA from human cell lines (lanes 1 to 11) or tissues (lanes 12 to 17) and hybridized with a radiolabeled LYSPl00 probe or a GADPH probe. Cell lines: HeLa, cervical carcinoma; K562, myelogenous leukemia; Hut78 and Jurkat, T cell; Nalm6, pre-B cell; and Raji, BJAB, MBB1, VDSO, WI2-NS, and ARH77, mature B cell.}
\end{figure}

\begin{table}[h]
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& 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 & 11 & 12 & 13 & 14 & 15 & 16 & 17 \\
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LYSPl00 & & & & & & & & & & & & -0.2 kb & & & & & & \\
GADPH & & & & & & & & & & & & -0.2 kb & & & & & & \\
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\caption{Expression of LYSPl00 mRNA in human lymphoid cell lines and tissues (Fig 1).}
\end{table}
Other LYS100 cDNAs account for the 0.9- and 1.6-kb mRNAs and are also most likely the products of alternative splicing (A.L.D., data not shown). The insertions in the LYS100-B cDNA alter the size of the predicted open reading frame: LYS100-A encodes a protein of 412 amino acids with a molecular weight of 46 kD, whereas LYS100-B encodes a protein of 882 amino acids with a molecular weight of 100 kD. The amino-terminal 158 residues of both LYS100 protein isoforms have 61% amino acid homology with the amino-terminal region of a previously described SP100 cDNA isosome30 (termed SP100-A in Fig 2A and B). Interestingly, the additional 400 amino acids found in LYS100-B but not in LYS100-A showed significant homology with another previously cloned protein, HNPP (Fig 2A and B). HNPP is highly inducible by interferon but has no known function.39

To identify potential SP100 cDNA isoforms that encoded a HNPP domain, we screened a HeLa cDNA library using a

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**Fig 2.** Amino acid sequence of LYS100 and comparison to SP100. (A) Two LYS100 protein isoforms aligned with 2 SP100 protein isoforms and HNPP1 (Genbank accession no. L22343). Identical amino acids are in black and conservative amino acid changes in grey. (B) Schematic representation of regions of high sequence homology between LYS100 isoforms, SP100 isoforms, and HNPP1. Striped boxes indicate regions in LYS100, SP100, and HNPP with high amino acid similarity. Additional regions of similarity within these proteins include the PHD/TTC domain (gray box) and the bromodomain (checkered box).

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<tr>
<th>LYS100-A</th>
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**Table:**

- **LYS100**
  - Homology Regions
  - PHD/Bromo-TTC Domain

- **SP100**
  - Homology Regions
  - PHD/Bromo-TTC Domain

- **HNPP**
  - Homology Regions
  - PHD/Bromo-TTC Domain
NOVEL NUCLEAR STRUCTURES DEFINED BY LYSPI00

C

Fig 2. (cont’d). (C) Alignments of the PHD/TTC domain and bromodomain of LYSPI00-B with other PHD/TTC- and/or bromodomain-containing proteins from the database. The spacer region between PHD/TTC domains and bromodomains of LYSPI00-B and TIFI are also aligned to show the similarity in this region between the 2 proteins. Identical amino acids and highly conservative amino acid substitutions compared with LYSPI00-B are in black. References or accession numbers for the protein sequences: TIFI,24 MLL, 003164(SP); trx, P20659(SP); BR140, M91585(GB); hCBP, S39162(PIR); mCBP, S39161(PIR); p300, U01877(GB); dFSH, P13709(SP); ORFX, D26362(GB); BDF1, 218944(GB); GCN5, Q03330(SP); CCG1, P21675(SP); SPT7, L22537(GB); BRAHMA, P25439(SP); hBRG1, S66910(GB); hBRM, X72889(GB); SNF2, P22082(PI); STH1, M83755(GB); and P61, X90849(GB). SP, Swiss protein; GB, GenBank.

probe derived from the LYSPI00-B HNPP homology region. Based on the sequence of a novel HNPP-like clone isolated from this screen, PCR primers were designed that were used with SP100 primers to amplify a novel SP100 isoform from Raji cDNA. Sequencing of a full-length cDNA for this SP100 isoform, termed SP100-B, revealed that it is identical to SP100-A over the first 477 amino acids, but it has a carboxy-terminal extension that is highly similar to but shorter than the carboxy-terminal domain of LYSPI00-B (Fig 2A and B). Thus, the LYSPI00-B and SP100-B isoforms share amino acid similarity throughout the length of the predicted proteins (Fig 2B), suggesting that these two proteins had a common evolutionary precursor.

Further sequence analysis of the LYSPI00-B isoform revealed two additional domains found in a variety of transcriptional regulatory proteins: a PHD/TTC domain39,41 and a bromodomain42 (Fig 2B and C). The PHD/TTC domain is a cysteine-rich motif with the general structure, C4HC3, that is presumed to coordinate zinc. Interestingly, like LYSPI00-B, several coactivator proteins contain both a PHD/TTC domain and a bromodomain, including TIFI,43 CREB binding factor,44 and p300.45 The PHD/TTC domain and bromodomain of LYSPI00-B are most closely related to those of TIFI, a potential transcriptional coactivator for nuclear hormone receptors.46 In addition, the spacer regions between PHD/TTC domains and bromodomains of LYSPI00-B and TIFI are similar in length and have homology to each other (Fig 2C), suggesting that the carboxy termini of these two proteins may have related functions. Taken together, the presence of the PHD/TTC domain and bromodomain in LYSPI00-B suggests that LYSPI00-B may be involved in transcriptional regulation.

Subcellular localization of LYSPI00 protein. We next generated affinity-purified rabbit antibodies against LYSPI00 to determine if LYSPI00, like SP100, was localized in PML NBs. The anti-LYSPI00 antibodies were raised against the carboxy terminus of LYSPI00-A, since this region displayed little homology to SP100. These anti-
LYSPl00 antibodies should also recognize the LYSPl00-B isofom, since most of the LYSPl00-A carboxy terminus is shared by LYSPl00-B (Fig 2A and B). Specificity of the anti-LYSPl00 antibodies was tested by Western blot analysis of nuclear and whole-cell extracts (Fig 3). A set of protein isoforms was observed in cells that express LYSPl00 mRNA (ARH77 and Raji), but not in cells lacking LYSPl00 mRNA (K562 and REH). The LYSPl00 isoforms ranged in size from 65 to 150 kD and may represent the products of alternatively spliced LYSPl00 mRNAs or may include proteolytic degradation products. In vitro translation of LYSPl00-A and LYSPl00-B isoforms showed that the protein products migrate more slowly in sodium dodecyl sulfate gels than predicted by the DNA sequence, as previously observed for SP100 (A.L.D., data not shown). By this criterion, LYSPl00-A mRNA can account for the 65-kD LYSPl00 protein isoform seen on Western blots, and LYSPl00-B mRNA can account for the 150-kD LYSPl00 protein isoform. In addition, the 100-kD LYSPl00 protein appears to be encoded by another LYSPl00 cDNA isoform (A.L.D., data not shown). The lower–molecular-weight bands on the Western blots were detected in all cells regardless of LYSPl00 mRNA levels, and therefore represent cross-reacting protein species (Fig 3).

Next, subcellular localization of LYSPl00 was investigated using the affinity-purified anti-LYSPl00 antibodies for immunofluorescent staining and confocal laser microscopy of the MBBl human lymphoblastoid cell line and human peripheral blood B lymphocytes (Fig 4). LYSPl00 localized to five to 20 dot-like structures in the nucleus of MBBl cells (Fig 4A, D, and G). LYSPl00-associated dots were also detected in nontransformed human peripheral blood B cells (Fig 4I, 3 cells shown). On average, the number of LYSPl00-associated dots was significantly lower in peripheral blood B cells than in the Epstein-Barr virus–transformed cell line: one to three structures were seen per peripheral blood B cell, with some cells having no detectable dots. LYSPl00 was detected in nuclear dots regardless of whether the cells were fixed with paraformaldehyde, methanol, or acetone, and nuclear localization of LYSPl00 dots was confirmed by co-staining the cells with a dye specific for DNA (data not shown).

Specificity of LYSPl00 staining was demonstrated in two ways. First, the characteristic LYSPl00 nuclear dots were found only in LYSPl00-expressing cells (MBBl, VDOS, and ARH77), and not in cells that are negative for LYSPl00 mRNA (K562 and HeLa) (A.L.D., data not shown). As a second test of antibody specificity, HeLa cells were transfected with a eukaryotic expression vector for LYSPl00-B and then costained with the anti-LYSPl00 antibodies (Fig 5A, green staining) and with the 5E10 monoclonal anti-PML antibody (Fig 5A, red staining). Ten percent to 20% of the cells showed a dot-like nuclear staining with anti-LYSPl00 antibodies that was similar to the LYSPl00 staining observed in MBBl lymphoblastoid cells. Nuclear localization of LYSPl00 dots was confirmed by Nomarski differential-interference contrast microscopy (Fig 5B). The remainder of the cells, which presumably did not incorporate DNA during the transfection, did not show this LYSPl00 staining pattern. These specificity studies demonstrate that although anti-LYSPl00 antibodies detected minor cross-reacting protein species in all cell types by Western blot analysis, these cross-reacting proteins were not readily detectable in the immunofluorescence assay. Furthermore, the HeLa transfection experiment demonstrates that LYSPl00 nuclear structures can be generated in nonlymphoid cells and thus do not require other lymphoid-specific proteins for their formation.

Surprisingly, in transfected HeLa cells, LYSPl00 and PML staining patterns were largely nonoverlapping (Fig 5A). Similarly, the majority of LYSPl00 and PML nuclear structures in MBBl and peripheral blood B cells were not coincident (Fig 4C, F, I, and L). In contrast, SP100 and PML colocalized precisely in MBBl cells in nuclear structures with the characteristic appearance of PML NBs (Fig 4M to O), confirming previous results. We next tested whether the LYSPl00 nuclear dots coincided with other previously described subnuclear domains. LYSPl00 immunofluorescent staining was entirely nonoverlapping with the nuclear speckles detected with antibodies to the splicing factor, SC35, and the nuclear dots detected with antibodies to p80-coilin (data not shown). Thus, LYSPl00 was localized to subnuclear structures that are distinguishable from PML NBs, interchromatin granules and interchromatin fibrils (containing SC35), and coiled bodies (containing p80-coilin).

Interestingly, in approximately 5% of the MBBl cells, PML dots and LYSPl00 dots exactly coincided within a confocal optical slice (Fig 4C and I). In some cells, only one dot was detected for LYSPl00 and PML (Fig 4C), whereas in other cells LYSPl00 and PML coincided in more than one structure (Fig 4I). In some cells, the pattern of PML/LYSPl00 colocalization was also striking, in that the
Fig 4. Laser-scanning confocal microscopy of cells immunofluorescently stained with antibodies to LYSPl00 and/or PML (A to L) or with antibodies to SP100 and/or PML (M to O). A to L and M to O were derived from the human lymphoblastoid cell line, MBB1, with each row representing images obtained from a single MBB1 cell. J to L were derived from human peripheral blood B lymphocytes. Three representative cells are shown in each panel. Green fluorescence (LYSP100 or SP100) and red fluorescence (PML) were collected simultaneously and then separated and merged digitally. Colocalization of green and red fluorescence yields a yellow image.
Fig 6. Laser-scanning confocal fluorescent microscopy z-series of a MBB1 cell stained with anti-LYSP100-B antibody (green fluorescence) and anti-PML antibody (red fluorescence). Twenty optical slices along the z-axis are shown.
LYSP100 immunofluorescence appeared to be surrounded by a ring of PML immunofluorescence (Fig 4I). Based on an analysis of a z-series of 20 confocal optical slices through this cell (Fig 6). PML appeared to form a shell around a central LYSPlOO core. This observation rules out the possibility that colocalization of LYSPlOO and PML in these structures occurred simply by chance superposition.

Next, the relationship of SP100 dots to LYSPlOO dots was judged by costaining with a polyclonal mouse anti-SP100 antibody and the polyclonal rabbit anti-LYSPlOO antibody (Fig 7). In most cells, SP100 and LYSPlOO were detected in nuclear dots that did not overlap (data not shown). However, in some cells dot-like structures containing both SP100 and LYSPlOO were detected, and often a single nucleus contained several such structures (Fig 7). Approximately the same minor fraction of MBBl cells (~5%) showed LYSPlOO/SP100 colocalization as showed LYSPlOO/PML colocalization. Thus, in a minority of cells, LYSPlOO colocalized with PML or SP100. In some images, the colocalization was reminiscent of PML NBs (Fig 4I). However, a more precise definition of LYSPlOO-associated nuclear structures required the higher resolution afforded by immun-EM.

Immuno-EM characterization of LANDs. We used immuno-EM to visualize the ultrastructural morphology and subcellular localization of LYSPlOO-associated structures. After fixation and embedding of MBBl lymphoblastoid cells, thin sections were reacted with LYSPlOO antibodies alone (Fig 8), with both LYSPlOO and PML antibodies (Fig 9), or with both LYSPlOO and SP100 antibodies (data not shown). LYSPlOO antibodies were secondarily reacted with anti-rabbit IgG antibodies coupled to 10-nm gold particles, whereas PML or SP100 antibodies were detected with antibodies coupled to 15-nm gold particles. LYSPlOO antibodies decorated electron-dense structures that were round to irregular in shape. These structures ranged from 0.1 to 0.3 μm in diameter, and thus were smaller than PML NBs, which were 0.3 to 1.0 μm. The electron density of LYSPlOO-associated structures was often uneven, and in many instances they appeared to have a central core that was more electron-dense (Figs 8A and 9A). Specificity of LYSPlOO immunogold detection was checked in two ways. First, the characteristic electron-dense structures were not observed when the gold-labeled goat anti-rabbit IgG alone was used for detection. Second, electron-dense structures detected by anti-LYSPlOO antibodies were observed frequently in MBBl cells but only rarely in HeLa cells, as predicted by the preferential expression of LYSPlOO in lymphoid cells.

LYSPlOO-associated structures and PML NBs were morphologically distinct in several respects. First, LYSPlOO structures often appeared more electron-dense than PML NBs (Fig 9C and D). LYSPlOO structures were uniformly decorated by LYSPlOO antibodies (Fig 8A to D), whereas PML tended to localize over the outer-ring structure of PML NBs (Fig 9C and D). LYSPlOO therefore appears to be associated with a novel nuclear domain that is distinct from PML NBs and other well-characterized subnuclear structures. We therefore propose to name these subnuclear domains "LANDs," for LYSPlOO-associated nuclear domains.

In sections stained with both LYSPlOO and PML antibodies, most LANDs were found to stain only with LYSPlOO. Furthermore, most PML staining was detected in typical PML NBs. However, some LANDs were decorated with PML staining. Most of these LANDs were observed with 10-nm and 15-nm gold particles, indicating the presence of both LYSPlOO and PML, respectively (Fig 9). These PML and LYSPlOO containing structures appeared to have the characteristics of LANDs, as already described, rather than the characteristics of PML NBs. Indeed, LANDs that contained PML could not be readily distinguished by morphology from those that did not. The ratio of LYSPlOO to PML gold particles in the structures shown in Fig 9 is 2.0 ± 0.49. The relatively low variation in this ratio points to a consistency in the molecular composition of the LANDs to which both LYSPlOO and PML localize. In contrast to LYSPlOO, antibodies to SP100 stained characteristic PML NBs exclusively, and were not detected in structures with LAND morphology (data not shown).

EM provided several intriguing refinements for the characterization of subcellular distribution of LANDs. Most LANDs were observed within the nucleoplasm in the interchromatin space (Figs 8A and 9A and D). However, many LANDs were localized precisely at the nuclear envelope, sometimes on the nuclear face (Fig 9C) and in other cases on the cytoplasmic face (Figs 8B and C and 9B). Frequently, these LANDs were seen at the tip of deep invaginations of the nuclear envelope into the nucleus (Figs 8B and 9B). Approximately one fifth of the LANDs were observed in the cytoplasm and were morphologically indistinguishable from LANDs present in the nucleoplasm (Fig 8D). LANDs that contained PML showed a similar subcellular distribution compared with LANDs devoid of PML. In summary, morphologically similar LANDs were observed in the nucleo-
plasm, at the nuclear membrane, and in the cytoplasm, raising the possibility that LANDs are transported between these compartments.

DISCUSSION

We have cloned a novel lymphoid-restricted nuclear factor, LYSP100, that is homologous to SP100, a protein found in dot-like nuclear domains termed PML NBs. As analyzed by confocal immunofluorescence microscopy, LYSP100 was also localized in dot-like subnuclear structures, but, surprisingly, LYSP100 dots were generally nonoverlapping with PML NBs. By immuno-EM, LYSP100 was found in novel electron-dense globular structures that were morphologically distinct from PML NBs and other previously described subnuclear structures. Thus, LYSP100 molecularly defines a novel subnuclear domain, which we term LAND, for LYSP100-associated nuclear domain. Interestingly, in a minor population of LANDs, LYSP100 and PML did colocalize, as judged by immuno-EM. Our data thus reveal LANDs as a new class of nuclear structures associated with PML.

LYSP100 and SP100 genes likely evolved from a common ancestor, since they encode proteins that share several regions of homology. All LYSP100 and SP100 isoforms share a highly conserved 157-amino acid amino-terminal domain. In addition, one isoform of LYSP100, LYSP100-B, contains a carboxy-terminal extension of 470 amino acids that bears homology with HnPP, a nuclear phosphoprotein that is strongly induced following interferon treatment of cells. We used this LYSP100 isoform to identify a previously undescribed SP100 isoform, SP100-B, which also contains a carboxy-terminal HnPP domain. A common feature of SP100, HnPP, and PML is that they are strongly upregulated by interferon treatment of cells. By contrast, interferon treatment has only a modest (~twofold) effect on LYSPl00 mRNA expression (A.L. Dent, unpublished observations, September 1993).

Of particular note, LYSP100-B shares two homology regions with several transcriptional regulatory proteins, the PHD/TTC domain and the bromodomain. The PHD/TTC domain is defined by a characteristic arrangement of cysteine and histidine residues, CnHnC, and is a potential zinc-coordination motif. Two PHD/TTC domain proteins, CBP and p300, mediate transcriptional activation through the cAMP-responsive transcription factor, CREB. The PHD/TTC motif is also repeated four times in the Drosophila homoeotic protein, trithorax, a positive regulator of transcription that is responsible for maintenance of the transcription of homeotic selector genes. The MLL gene, a trithorax homologue translocated in a variety of human leukemias, has particularly high sequence homology with trithorax in the PHD/TTC region.

The closest homologue to LYSP100-B in the PHD/TTC domain is TIF1, a potential coactivator protein for nuclear hormone receptors. TIF1 binds to the AF-2 activation domains of nuclear hormone receptors in a ligand-dependent fashion and can stimulate transcription through these receptors in yeast. Interestingly, TIF1 displays a more extended homology with LYSP100-B that includes the bromodomain and the spacer region between the two domains, suggesting that these two domains perform related functions in the two proteins. In addition to TIF1, the coactivator proteins, CBP and p300, also contain both a bromodomain and a PHD/TTC domain. The bromodomain is also found in CCG1/ hTAFI1250, a protein that is tightly associated with TATA binding factor and is required for the function of certain transcriptional activation domains. A large, functionally distinct class of bromodomain proteins are homologues of the Drosophila homeotic protein, Brahma, and the yeast SWI12/SNF2 protein, and appear to contribute to a multiprotein complex that disrupts chromatin. Although the precise function of the bromodomain is not yet clear, deletion of this domain in the yeast transcriptional adaptor protein, GCN5, impairs its ability to activate transcription mediated by certain activation domains. These sequence homologies provide provocative, albeit preliminary, indications that LYSP100 may participate in transcriptional regulation. The lymphoid-restricted expression of LYSP100 further suggests that LYSP100 does not perform a merely "housekeeping" function, but instead may act to regulate transcription in a cell type-specific fashion.

LANDs were distinct in location and morphology from other subnuclear structures that have been molecularly defined, including interchromatin granules, perichromatin fibris, coiled bodies, and PML NBs. EM showed LANDs to be round or irregularly shaped electron-dense structures ranging in size from 0.1 to 0.3 μm. Three-dimensional confocal image plane reconstruction of LANDs showed them to be solid, roughly spherical structures (A.L.D., J.Y., and L.M.S., unpublished data, March 1994). Thus, the morphology of LANDs as revealed by immunofluorescence or EM is not notably distinctive, and consequently, this novel nuclear subdomain could not have been appreciated without the cloning and characterization of LYSP100. At present, since LYSP100 is a lymphoid-restricted protein, it is not possible to convincingly demonstrate that LANDs exist in nonlymphoid cells. However, in this regard, it is noteworthy that when LYSP100-B was expressed in HeLa cells by transfection, LYSP100 antibodies detected dot-like nuclear structures by immunofluorescence that were indistinguishable from LANDs detected in lymphoid cells. One interpretation of this result is that the ectopically expressed LYSP100 was incorporated into LAND-like structures that preexisted in HeLa cells. Alternatively but seemingly less likely, LYSP100 might organize de novo LANDs within HeLa cells.

The ultrastructural location of LANDs may provide some intimation of their function. The greatest number of LANDs was observed in the nucleoplasm, where they could be detected in the interchromatin regions (Figs 7 and 8) and in the nucleolar regions (data not shown). However, in addition, many LANDs were directly touching the nuclear envelope, both on the nuclear and cytoplasmic face, and approximately one fifth of the LANDs were in the cytoplasm proper. Intriguingly, LANDs were frequently observed at the tip of deep invaginations of the nuclear membrane (Figs 7B and 8B). It is unclear how such invaginations in the nuclear envelope are formed and maintained, but they could conceivably represent functional specializations of the nuclear enve-
lope. These observations suggest the possibility that LANDs may traverse the nucleus to the cytoplasm, or vice versa. In the former case, LANDs might be multisubunit structures that, like ribosomes, are assembled in the nucleus but function in the cytoplasm. Alternatively, LANDs might be directly involved in the transport of macromolecules such as RNA from the nucleus to the cytoplasm. On the other hand, LANDs, like snRNPs, may be assembled in the cytoplasm and then imported into the nucleus. Once in the nucleus, LANDs may serve as sites where specialized nuclear functions take place, or they may be storage areas for proteins that can be released from LANDs to function elsewhere in the nucleus.

Finally, our data provide evidence for the association of PML with a subnuclear structure other than the PML NB. PML-containing LANDs were found in the nucleoplasm, at the nuclear membrane, and in the cytoplasm, and were morphologically indistinguishable from LANDs lacking PML. Previously, PML has been localized by immunofluorescence to the cytoplasm of some cells, yet PML NBs have only been described by EM in the nucleoplasm. Therefore, some of the previously described cytoplasmic PML may be attributable to LANDs. Furthermore, although the majority of PML in the nucleus is associated with PML NBs, some of the nuclear dots detected by immunofluorescence with anti-PML antibodies could represent LANDs. Recent studies have demonstrated that PML can suppress the growth and malignant transformation of cells, and can repress the activity of certain promoters in transient transfection assays. The present results suggest that LANDs should be taken into consideration in models of PML function.

One possibility raised by the presence of PML in both LANDs and PML NBs is that LANDs might be precursors in
the biogenesis of PML NBs. Conceivably, PML-containing LANDs might coalesce to generate the ring-like PML NBs. In this regard, the confocal immunofluorescence image of LYSPL100 surrounded by a ring of PML (Fig 4I) is especially intriguing. Given the annular nature of PML NBs by EM, it seems possible that the ring-like structures in Fig 4I are unusually large PML NBs. Thus, in a small percentage (<5%) of cells, LYSPL100 may be located in PML NBs. By immuno-EM, we were unable to detect LYSPL100 in characteristic PML NBs, but this failure may simply reflect the difficulty of detecting rare events by EM. If LYSPL100 is indeed present in a small subpopulation of PML NBs, it seems possible that LANDs are precursors to these structures.

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