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Identification and Characterization of Thymus LIM Protein: Targeted Disruption Reduces Thymus Cellularity

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We have identified a novel LIM gene encoding the thymus LIM protein (TLP), expressed specifically in the thymus in a subset of cortical epithelial cells. TLP was identified as a gene product which is upregulated in a thymus in which selection of T cells is occurring ($Rag^{-/-}$ OT-1) compared to its expression in a thymus in which selection is blocked at the $CD4^+$ $CD8^+$ stage of T-cell development ($Rag^{-/-}$ $Tap^{-/-}$ OT-1). TLP has an apparent molecular mass of 23 kDa and exists as two isomers (TLP-A and TLP-B), which are generated by alternative splicing of the message. The sequences of TLP-A and TLP-B are identical except for the C-terminal 19 or 20 amino acids. Based on protein sequence alignment, TLP is most closely related to the cysteine-rich proteins, a subclass of the family of LIM-only proteins. In both medullary and cortical thymic epithelial cell lines transduced with TLP, the protein localizes to the cytoplasm but does not appear to be strongly associated with actin. In immunohistochemical studies, TLP seems to be localized in a subset of epithelial cells in the cortex and is most abundant near the corticomedullary junction. We generated mice with a targeted disruption of the *Tlp* locus. In the absence of TLP, thymocyte development and thymus architecture appear to be normal but thymocyte cellularity is reduced by approximately 30%, with a proportional reduction in each subpopulation.

T-cell development requires many interactions between thymocytes and thymic stromal cells. As thymocytes develop, they begin as $CD4^-$ $CD8^-$ double-negative (DN) cells which proliferate and rearrange their T-cell receptor (TCR) beta chain genes. The DN thymocytes, which express a pre-TCR, progress to $CD4^+$ $CD8^+$ double-positive (DP) cells, which rearrange TCR alpha chain genes and express TCR. A small percentage of the DP thymocytes are positively selected into the CD4 single-positive (SP) or CD8 SP lineage. Fully mature SP cells then emigrate to the periphery. Thymic stromal cells, a heterogeneous population of cells including epithelial cells, fibroblasts, mesenchyme cells, and the bone marrow-derived dendritic cells (DCs) and macrophages, are involved at each step in T-cell development (reviewed in references 4, 54, and 74). The DN-to-DP transition requires cell-cell interactions between early precursor thymocytes and both thymic epithelial cells (TECs) and fibroblasts (3, 57). In addition, cytokines that are necessary for the early expansion of DN cells and at later thymocyte developmental stages are provided by TECs (21, 46). For example, interleukin-7 (80) and stem cell factor (52) each appear to have an important role in the proliferation of thymocytes (29, 32, 49, 76). Positive selection of thymocytes is mediated by cortical TECs (cTECs) expressing the major histocompatibility complex (MHC)/peptide ligand for TCR (3, 63). cTECs are unique in this capacity, providing more than just the selecting ligand, since other cell types expressing the ligand are unable to substitute for them (5, 16, 47). DCs in the medulla and at the corticomedullary junction mediate negative selection of thymocytes with too high an affinity for self peptide/MHC ligand (44, 48, 53, 69). The medullary epithelium also appears to play a role in negative selection but is less efficient than DCs (26, 30, 65). The interactions between DP thymocytes and thymic stromal cells are critical to ensure that a sufficient T-cell repertoire with the appropriate degree of affinity for self develops.

TECs constitute the majority of thymic stromal cells and can be divided into three main classes based upon location in the thymus: subcapsular, cortical, and medullary. cTECs and medullary TECs (mTECs) are phenotypically distinguishable from each other by morphology and immunohistology using monoclonal antibodies (Abs) (reviewed in reference 11). For example, 6C3, also known as BP-1, stains only cTECs (1), while ERTR5 stains only mTECs (75). Within the cTEC population, there appears to be further heterogeneity based on staining with several monoclonal Abs. For example, 6C3 (1) and NLDC145 (42), also known as DEC-205 (35), each stain only a subset of cTECs. There appear to be at least two types of cTECs based on expression of keratin 5 (K5) and K8 (39). The majority of cTECs express K8 but not K5, while a minor subset express both. It is not clear whether phenotypically different cTECs represent differentiated cell types with specialized functions or cells undergoing different responses to thymocytes in different microenvironments.

Much is known about the origins of TECs, but little is known about the molecular mechanisms governing their development and differentiation into functional cells. It is clear, however, that development of TECs and organization of the thymus into cortex and medulla are dependent on thymocyte development (reviewed in reference 62; 4, 54, 74). Development of mTECs and formation of an organized medulla depend on the pres-

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ence of $\alpha\beta TCR^+$ thymocytes, as seen in SCID (66, 67), $RAG^{-/-}$ (51), and $TCR\alpha^{-/-}$ (58, 60) mice. In addition, there are data to suggest that the organization of the medullary epithelium is influenced by signals from thymic vasculature (6). The three-dimensional organization of a thymic cortex into microenvironments capable of promoting T-cell development appears to depend on progression of DN thymocytes from a $CD44^+$ $CD25^-$ phenotype to a $CD44^-$ $CD25^+$ phenotype (31, 73). At the molecular level, a number of transcription factors, including whn, relB, Pax9, Hoxa3, and Pax1, have been shown to play important roles in thymus organogenesis and TEC development (13, 15, 55, 59, 68). For the most part, however, the molecular mechanism for development of TECs is unknown.

LIM proteins are known to play a number of important roles in development and differentiation (reviewed in reference 10). They are defined by the presence of a LIM domain, which consists of two tandemly repeated zinc fingers with the following consensus sequence: $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-23}CX_{2-3}$(C/H/D) (64). The LIM domain mediates protein-protein interactions which are important for such processes as gene regulation and cytoskeletal organization (10). One subclass of LIM proteins, the LIM-only proteins, do not have a homeodomain. Among the LIM-only proteins are the cysteine-rich proteins (CRPs). Three family members have been identified in vertebrates: CRP1, CRP2, and CRP3/muscle LIM protein (MLP) (8, 28, 33, 36, 43, 50, 56, 78, 79, 83). These three proteins are highly similar to each other in sequence and structure but have distinct patterns of expression. Functionally, MLP has been shown to promote myogenesis (8) through its interaction with MyoD (40) and to be required for normal cardiomyocyte cytoarchitecture and function (9).

To identify novel genes involved in positive selection of thymocytes, we performed PCR-based subtractive hybridization between a thymus in which there was a high degree of positive selection and a thymus in which selection is blocked at the DP stage of development (37). Mice with highly selecting thymuses expressed a MHC class I (MHC-I)-restricted transgenic TCR (OT-1) on a $RAG2^{-/-}$ background. Thymocytes in these mice are selected primarily into the CD8 lineage. Mice with nonselecting thymuses expressed the same TCR but on a $RAG2^{-/-}$ $TAP1^{-/-}$ background. In the absence of TAP, the selecting ligand is unavailable and thymocytes do not progress beyond the DP stage.

We identified a novel gene which is upregulated in the selecting thymus compared to its expression in the nonselecting thymus. Based on the homology of its product to the CRPs and its specific expression in the thymus, we named this gene the thymus LIM protein (*Tlp*) gene and propose that its product is a novel member of the CRP family of LIM-only proteins. We identified the TLP protein and showed that, within the thymus, TLP is expressed in a unique pattern in a subset of cTECs, most abundantly near the corticomedullary junction. To determine the requirement for TLP in thymocyte development, we generated mice with a targeted disruption of the *Tlp* locus. These mice appeared to have normal thymocyte development and thymus architecture but had a 30% decrease in thymocyte cellularity compared to littermate controls.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and Taconic Farms (Germantown, N.Y.). $RAG2^{-/-}$ $TAP1^{-/-}$ OT-1 and $RAG2^{-/-}$ OT-1 mice were bred in our laboratory as previously described (37). The TCli TCR transgenic mice (81) and the HY TCR transgenic mice (38) have been described previously. The $TLP^{-/-}$ mice were generated by targeted disruption of the TLP locus in 129Sv embryonic stem (ES) cells using standard methodology (see below) (61). $TLP^{+/-}$ ES cells were injected into C57BL/6 blastocysts. Founder lines were established by breeding chimeric male founders to both C57BL/6 and 129Sv females. The $TLP^{+/-}$ F_1 progeny were then interbred to generate $TLP^{+/+}$, $TLP^{+/-}$, and $TLP^{-/-}$ mice.

Targeted disruption of the TLP gene locus. A BAC clone containing the entire TLP gene locus was isolated from a mouse 129 genomic DNA library (Genome Systems, Inc., St. Louis, Mo.). By restriction enzyme mapping and Southern blot analysis, a 12-kb *Kpn*I genomic DNA fragment containing the entire TLP coding sequence was identified and subcloned into pBlueScript (Stratagene). A 2.1-kb fragment corresponding to the region immediately upstream of and including the TLP gene start codon was subcloned into targeting vector pSABGalpgkneolox2PGKDTA (a kind gift from Philippe Soriano). The 3′ arm was a 3.6-kb *Nhe*I fragment of genomic DNA downstream of the TLP gene. Two separate ES cell lines were used for the homologous recombination: AK7 (kind gift from Philippe Soriano) and TC1 (20). ES cells were grown on mitomycin C-treated SNL feeder cells (61) and transfected by electroporation with the linearized targeting construct DNA. Transfected cells were selected in 300 μg of G418/ml, and colonies were isolated after 10 days of selection. Colonies with homologous recombination were identified by PCR using a primer annealing to a region upstream of the 5′ arm (5′CTGCTTCTACCTTCCAAGGAC3′) and a primer annealing to the β-galactosidase (β-Gal) gene (5′AGGGGACGACGA CAGTATC3′). Targeted recombination resulting in removal of TLP gene exons 1 to 8 (including the entire coding sequence except for that encoding the first amino acid) was confirmed by Southern blot analysis of genomic DNA.

Commercial antibodies and flow-cytometric analysis. Conjugates of the following monoclonal Abs were purchased from Pharmingen (San Diego, Calif.) and Caltag Laboratories (Burlingame, Calif.) and used in flow cytometry: anti-CD3ε, anti-CD4, anti-CD8α, anti-CD45R (B220), anti-CD11b (Mac-1), anti-$I\text{-}A^b$ (MHC-II), anti-CD69, anti-CD24 (heat-stable antigen), anti-CD11c, anti-CD80, anti-CD86, F4/80, and DEC-205. Anti-human c-myc and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Flow cytometry of thymocytes was performed as described previously (37).

Isolation of TLP by PCR-based subtractive hybridization and cloning of the full-length cDNA. Isolation of thymus RNA from mice with selecting ($Rag2^{-/-}$ OT-1) or nonselecting ($Rag2^{-/-}$ $Tap1^{-/-}$ OT-1) thymuses, generation of cDNA, and performance of PCR-based subtractive hybridization were as previously described (37). From this procedure, a fragment of TLP cDNA was isolated and used to probe a mouse thymus cDNA phage library (generously provided by James Allen). Four independent cDNA clones were isolated, sequenced, and used to generate a contig. The consensus sequence contained a portion of the TLP gene open reading frame (ORF) interspersed with untranslated sequence. We used rapid amplification of cDNA ends (Clontech, Palo Alto, Calif.) to clone the 5′ and 3′ ends of the TLP gene using thymus stromal cell cDNA and then cloned the full-length cDNA using the following primers, in which the underlined sequences anneal to the 5′ and 3′ ends, respectively, of the TLP gene: 5′AAG GATCCGCGGCCGCTGAAGAACCATGAGCTGGAC3′ and 5′AAAAAAC TCGAGGTCGACTTTTTGGGAATCAGTCACCATTTTA3′. The ~1-kb PCR product was subcloned, sequenced, and found to consist of two different cDNAs: TLP-A (965-bp) and TLP-B (1,024-bp) cDNA.

RNA isolation and Northern blot analysis. Total RNA was isolated using RNA STAT-60 (Tel-Test, Friendswood, Tex.), and mRNA was prepared using a FastTrack kit (InVitrogen, Carlsbad, Calif.). Northern blot analysis was performed essentially as described previously (37). The probes used were fragments of or complete cDNAs of mouse TLP, EF1α, CD4, and $I\text{-}A^b$ (MHC-II) and chicken GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Probes were radiolabeled using a random primer labeling kit (Invitrogen Life Technologies) and [α-^{32}P]dATP (Dupont, Boston, Mass.). For the Northern analysis of thymocytes versus stromal cells, thymocytes were dissociated from stroma by pressing a thymus through a Nytex filter. Undissociated stromal material was collected from inside the filter. Total RNA was isolated from the enriched fractions. Murine cell lines used for Northern analysis were mammary epithelial cell line C57/MG (70) and thymic epithelial cell lines 427.1 (22), 1308.1 (22), 6.1.7 (22), Z210R.1 (24), 100.4 (22), and TE-71 (25).

Generation of anti-TLP antiserum and immunoblot analysis. A glutathione *S*-transferase (GST)–TLP fusion protein containing TLP amino acids (aa) 78 to 115 was used to immunize New Zealand White rabbits (R and R Rabbitry, Stanwood, Wash.). A portion of the TLP cDNA was cloned into pGEX-2T, and expression and purification of the fusion protein were performed according to the manufacturer's instructions (Pharmacia Biotech). The antiserum was affinity purified using a Sepharose column to which the GST-TLP fusion protein was coupled. To remove GST-reactive Abs, the eluate was then immunoabsorbed with GST-coupled Sepharose, and the flowthrough was used for immunoblot analysis and immunofluorescence microscopy.

Whole-cell lysates were prepared using lysis buffer (1% NP-40, 100 mM Tris [pH 8.0], 150 mM NaCl) and quantitated by a Bradford assay. Equal amounts of protein were loaded per lane for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. Each step of the immunoblot analysis was performed for 1 h at room temperature. Detection of secondary Abs was performed using an ECL kit (Amersham, Arlington Heights, Ill.) followed by autoradiography (Kodak, Rochester, N.Y.). Affinity-purified anti-human c-myc and affinity-purified anti-TLP antiserum were each used at 1:100. HRP-conjugated secondary Abs goat anti-mouse IgG and goat anti-rabbit IgG were used at 1:1,000.

Retrovirus-mediated expression of TLP. The *Tlp* gene was cloned into the PMI vector, retroviruses were generated using a packaging cell line, and infections were performed as previously described (19). A 10-aa sequence (EQKLISEEDL) from human c-myc was added to the C termini of TLP-A and TLP-B by using primers 5′TTTTTTGTCGACGAATTCTCACAGGTCCTCTTCTGAGATCAGTTTTTGTTCTTTGGATCTTATGTCCACTGGGTC3′ and 5′TTTTTTGTCGACGAATTCTCACAGGTCCTCTTCTGAGATCAGTTTTTGTTCCACCACAAGGGAGGTTGTCC3′, respectively, together with 5′AAAAAAGCGGCCGCGGAATTCACCATGAGCTGGACTTGTCCGCGTT3′ in PCR. COS cells were infected with the retrovirus containing the c-myc-tagged versions of TLP-A and TLP-B (TLP-myc). TEC lines (427.1 and Z210R.1) were infected with TLP-A, TLP-B, or PMI vector alone. Infected cells were enriched by electronic sorting for cells expressing hCD2, a reporter gene in PMI.

Immunofluorescence confocal microscopy. Immunofluorescence microscopy of cultured cell lines was performed essentially as described previously (37). Uninfected and infected cells were fixed in 4% formalin, permeabilized in 0.1% Triton X-100, and stained using affinity-purified anti-TLP at 1:100 and rhodamine-phalloidin (Molecular Probes, Eugene, Oreg.) at 1:600. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG was used to visualize anti-TLP staining.

For analysis of thymus sections, immunohistochemistry was performed essentially as described previously, with the exception of using an alkaline phosphatase-linked fluorescence detection system (23). Briefly, thymus lobes were frozen in O.C.T. compound (Tissue-Tek; Miles, Inc., Elkhart, Ind.) in a dry ice-ethanol bath and stored at −80°C. Seven-micrometer-thick cryosections were air dried overnight and fixed in acetone for 10 min at −20°C prior to staining. The primary Abs used were affinity-purified anti-TLP at 1:100 and the rat monoclonal hybridoma supernatants 6C3 (1), ERTR5 (75), and NLDC145 (42), each at 1:1. Visualization of anti-TLP staining was performed using a Vectastain ABC-AP kit and Vector Red (Vector Laboratories, Burlingame, Calif.). Visualization of the rat monoclonal Abs was performed using FITC-conjugated goat anti-rat IgG. Levamisole (1.25 mM) was included during incubation with the Vector Red alkaline phosphatase to inhibit endogenous alkaline phosphatase activity. The stained cells and tissue sections were mounted and viewed by confocal microscopy using a 10× objective or a 40× or 100× oil lens with an MRC-1024 system (Bio-Rad, Hercules, Calif.) equipped with LaserSharp software and mounted on an Axiovert TV microscope (Carl Zeiss, Inc., Thornwood, N.Y.). Images were electronically overlaid using Adobe Photoshop or National Instititutes of Health Image software (public domain; http://rsb.info.nih.gov/nih-image/download.html) with Bio-Rad plugins.

RESULTS

The *Tlp* gene is a novel LIM-only gene. To identify genes whose expression was regulated during thymocyte development, we performed PCR-based subtractive hybridization using cDNA generated from thymuses of mice in which a high degree of T-cell selection occurs ($RAG2^{-/-}$ OT-1) and in which selection is blocked at the $CD4^+$ $CD8^+$ DP stage ($RAG2^{-/-}$ $TAP1^{-/-}$ OT-1). We refer to these mice as selecting and nonselecting, respectively. We used whole-thymus tissue rather than isolated thymocytes to identify genes whose expression could be developmentally regulated in thymocytes or stromal cells. In the selecting-minus-nonselecting direction of subtraction, we cloned a 1.3-kb *Rsa*I fragment of TLP. In BLAST (2) searches, this DNA fragment was not identical in sequence to any genes in the NCBI nonredundant database but did match a small number of mouse expressed sequence tags (ESTs) from thymus (GenBank accession no. BF730098 and BF720933), mammary gland (GenBank accession no. AA671952), and an eight-cell-stage embryo (GenBank accession no. AU020984). In searching the human genome sequence, we identified eight regions on human chromosome 6 which were 88 to 94% identical to contiguous regions of the mouse TLP gene sequence (2). This region on chromosome 6 is not currently assigned a gene name. Although the matching sequence is incomplete, the high degree of sequence identity at both the nucleotide and amino acid level suggests that this sequence is the human homologue of the mouse TLP gene (data not shown).

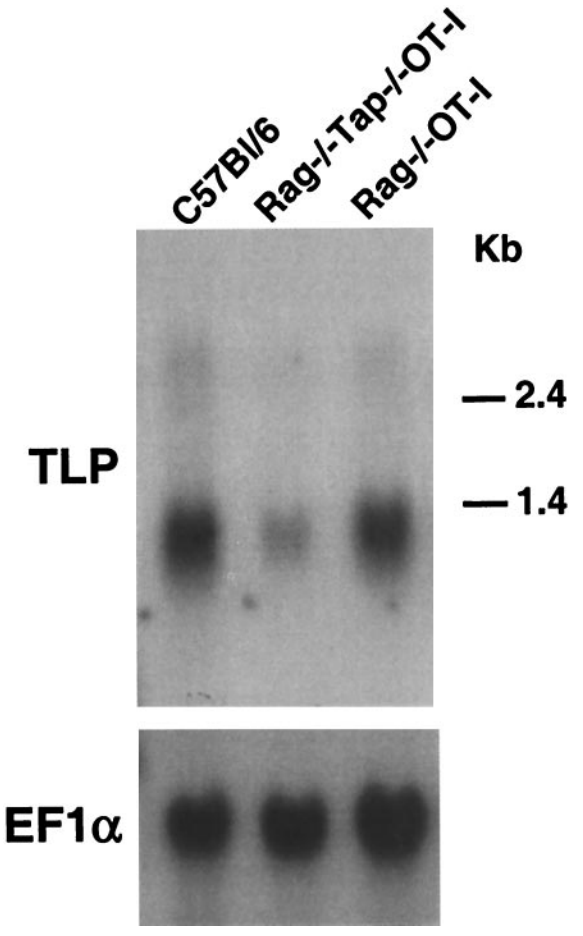


FIG. 1. TLP is upregulated in a selecting thymus compared to its expression in a nonselecting thymus. Whole-thymus total RNA (10 μg) from selecting (C57BL/6 and $Rag2^{-/-}$ OT-1) and nonselecting ($Rag2^{-/-}$ $Tap1^{-/-}$ OT-1) mice were used for Northern analysis. The blot was probed for TLP and normalization control EF1α.

To confirm that TLP was upregulated in a selecting thymus compared to its expression in the nonselecting thymus, we

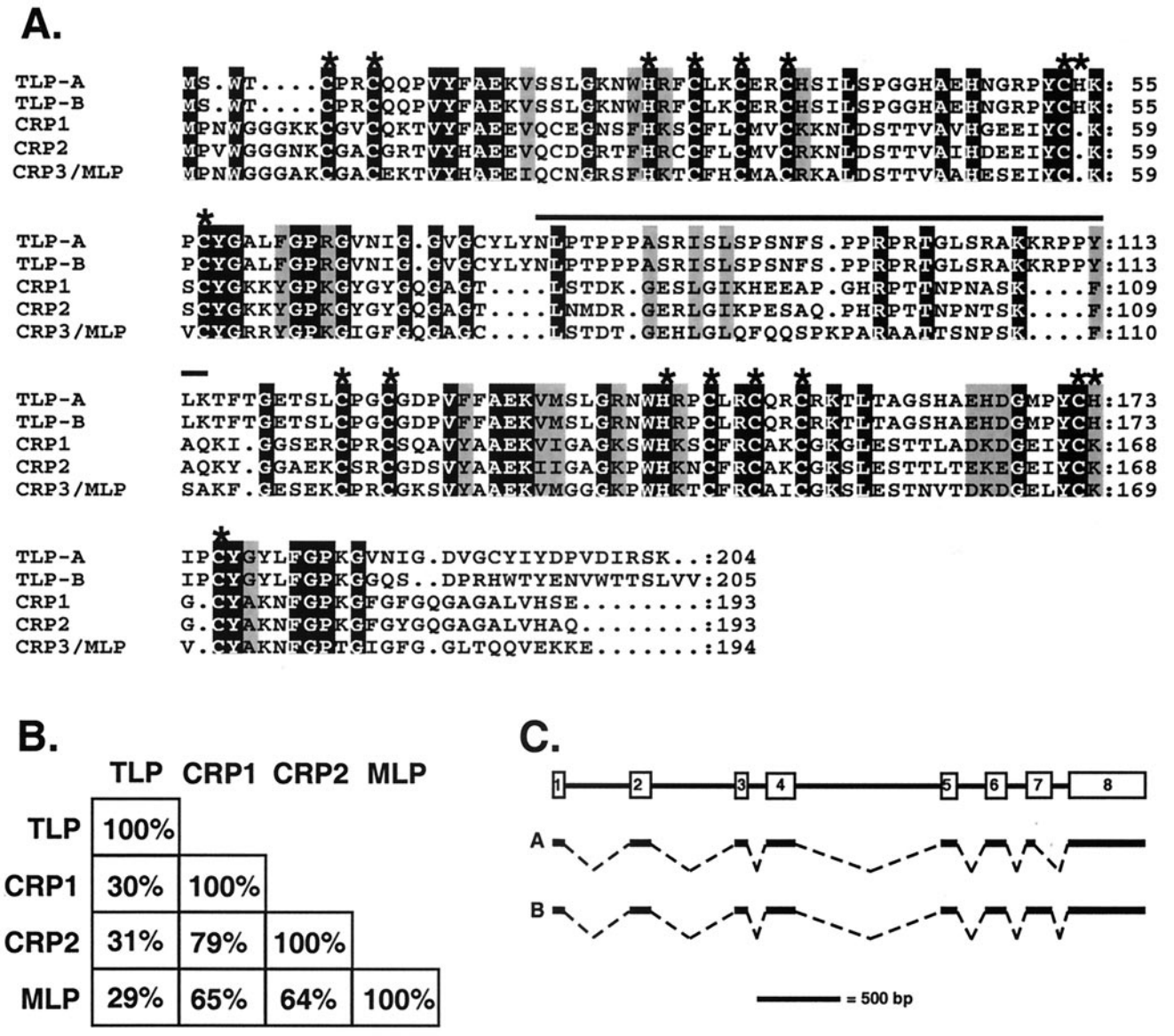


FIG. 2. TLP is a novel LIM-only protein closely related to the CRPs. (A) Alignment of TLP-A, TLP-B, CRP1, CRP2, and CRP3/MLP. The amino acid sequences were aligned using CLUSTALW (71). Conserved amino acids are shaded: black for identical residues and gray for similar residues. *, C and H residues of the LIM domains. The region of TLP used to generate anti-TLP antiserum is overlined. (B) Percentages of amino acid identity among TLP-A or -B, CRP1, CRP2, and CRP3/MLP. (C) TLP exists as two isomers (TLP-A and TLP-B), which are generated by alternative splicing. By aligning the sequences of two different cDNAs encoding TLP with the genomic DNA sequence, the structure of the TLP gene was determined. The TLP gene has eight exons and seven introns. TLP-A and TLP-B appear to be generated due to alternative splicing using two different splice donor sites in exon 7. The GenBank accession numbers for TLP-A and TLP-B cDNAs are AF367970 and AF367971, respectively. The GenBank accession number for the TLP gene is AF367972.

examined expression in C57BL/6 mice with both selecting and nonselecting thymuses by Northern analysis (Fig. 1). We found that TLP was significantly upregulated in both wild-type and selecting thymuses, compared to its expression in the nonselecting thymuses. The primary transcript detected in each thymus sample was ~1 kb. In addition, there was a smear of bands from 1 to 3 kb, indicative of incompletely or alternatively spliced transcripts.

In cloning the full-length *Tlp* cDNA, two isomers (TLP-A cDNA, 959 bp; TLP-B cDNA, 1,018 bp) were isolated with equal frequency. The cDNAs each contained a single ORF encoding predicted proteins of 204 and 205 aa, respectively (Fig. 2A). We predict that the start codon is that encoding the first Met residue at amino acid position 3 based on a strong Kozak sequence surrounding it (41) and the presence of an in-frame stop codon upstream. TLP-A and TLP-B were identical from aa 1 to 185 and differed in the remaining C-terminal sequence.

In BLAST (2) searches of the protein sequence database, TLP-A and TLP-B each had the highest degree of amino acid identity (>29%) with each of the three CRP family members: CRP1, CRP2, and MLP (Fig. 2A and B). TLP is not as similar

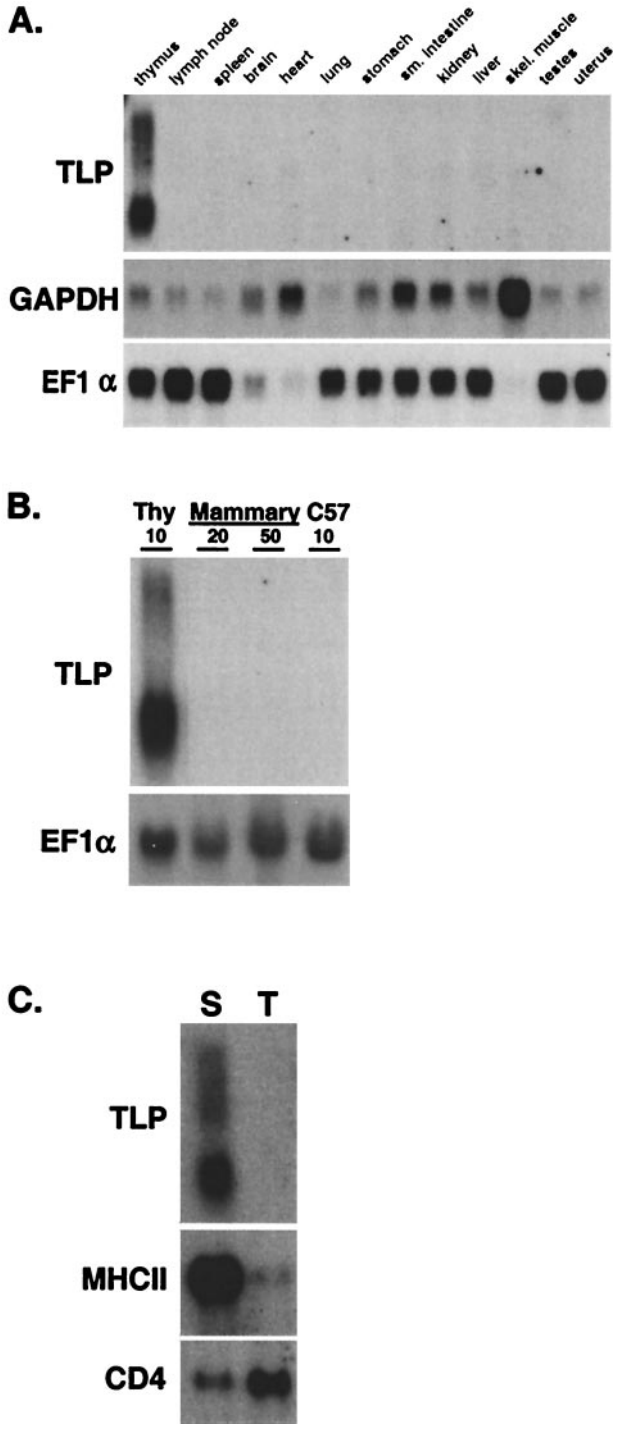


FIG. 3. TLP is thymus specific and is expressed only in the stromal cell compartment. (A) Northern analysis of several adult mouse tissues. mRNA (2 μg) harvested from whole tissue was used in Northern analysis with probes for TLP and normalization controls GAPDH and EF1α. sm., small; skel., skeletal. (B) Total RNA from whole thymus (Thy; 10 μg), mammary tissue from an 8-week-old lactating mouse (20 or 50 μg, as indicated), and a mammary epithelial cell line (C57; 10 μg) was used in Northern analysis with probes for TLP and EF1α. TLP was not detected in mammary tissue or C57 cells, even after a long exposure. (C) mRNA (2 μg) from thymic stromal material (S) and thymocytes (T) was used in Northern analysis with probes for TLP, MHC-II (I-A^b), and CD4. MHC-II (not expressed in thymocytes) and CD4 (not expressed in thymic epithelial cells) are controls for cross contamination.

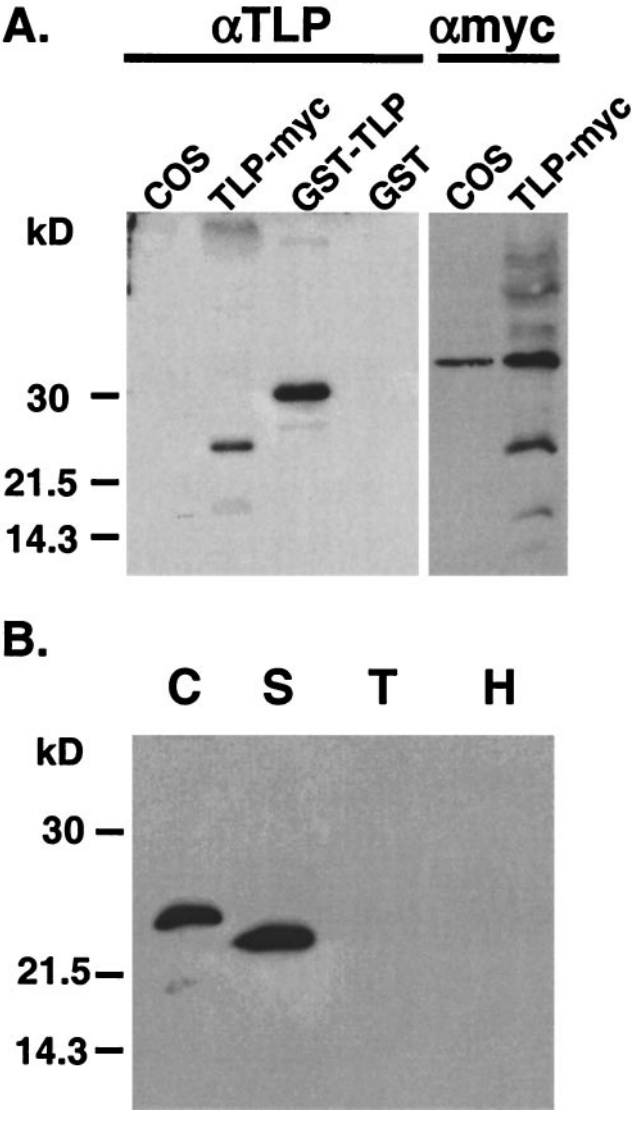


FIG. 4. Identification of TLP. A GST-TLP fusion protein containing TLP aa 78 to 115 (Fig. 2A) was used to immunize rabbits. The rabbit antiserum was affinity purified and used for Western analysis. (A) Equal amounts of whole-cell lysate prepared from control COS cells or COS cells infected with a retrovirus encoding TLP-A (TLP-myc) were used in SDS-PAGE and probed with either the affinity-purified anti-TLP antiserum (αTLP) or anti-myc (αmyc) on two separate blots. The bacterially expressed GST-TLP fusion protein (1 ng) and GST protein (1 ng) were included as controls for the specificity of the antiserum. (B) Equal amounts of extracts from thymic stromal cells (S), thymocytes (T), and heart tissue (H) were used in SDS-PAGE and probed with anti-TLP affinity-purified antiserum. Protein lysate from COS cells transduced with C-terminally myc-tagged TLP (C) was used as a positive control. The addition of the 10-aa myc tag results in a slower migration.

in amino acid sequence to the CRPs as they are to each other. However, like each of the CRP family members, TLP has two LIM domains, each of which is followed by a glycine-rich region. As in the CRP family members, the first zinc finger within each LIM domain of TLP is of the C-C-H-C type (Fig. 2A). The second zinc finger in each TLP LIM domain, however, is of the C-C-C/H-C type, differing from those in CRP1,

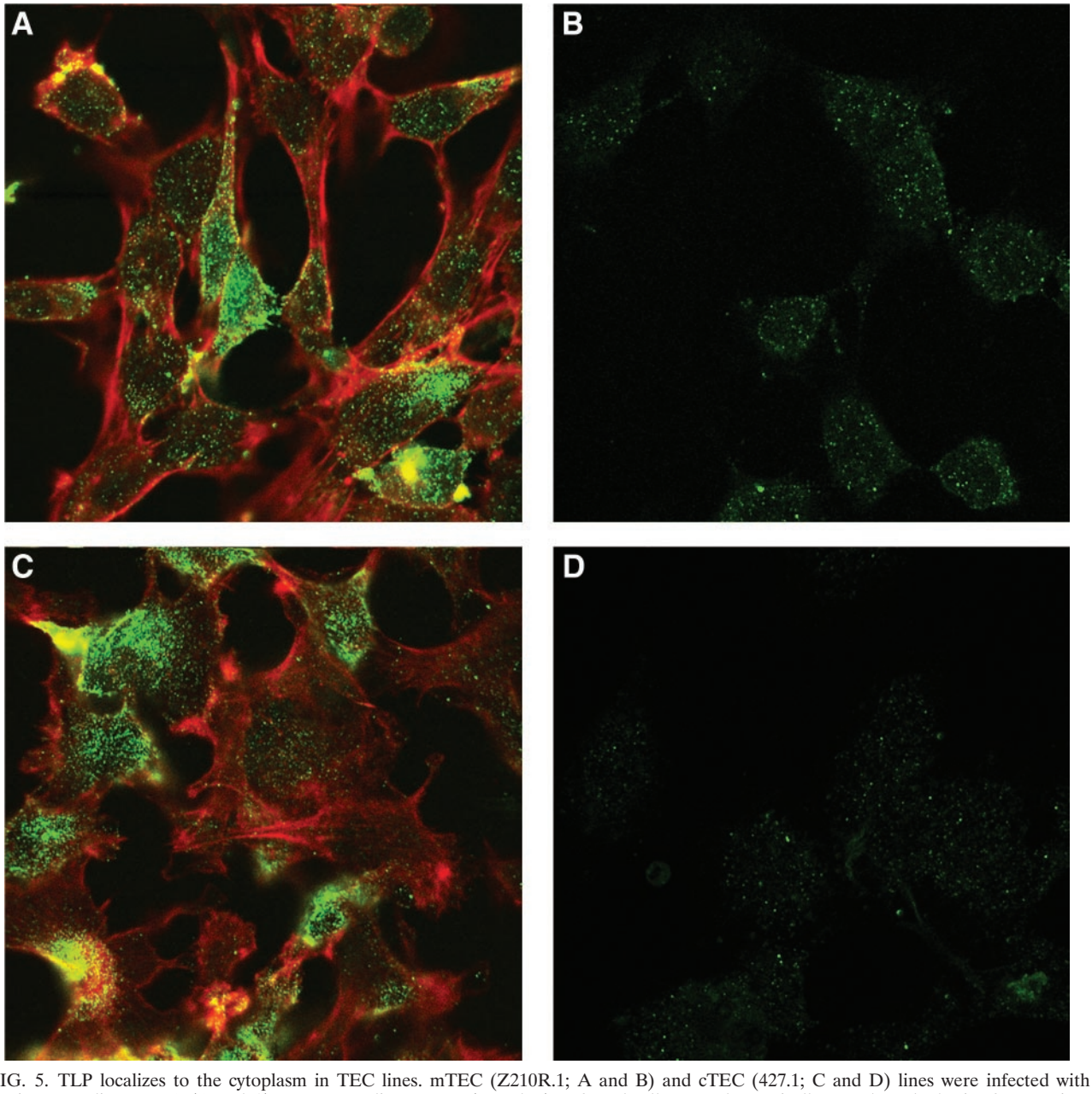


FIG. 5. TLP localizes to the cytoplasm in TEC lines. mTEC (Z210R.1; A and B) and cTEC (427.1; C and D) lines were infected with a retrovirus encoding TLP-A (A and C) or not encoding TLP-A (B and D). Infected cells were electronically sorted on the basis of expression of a reporter gene (hCD2), cultured, and used for immunofluorescence confocal microscopy. After culturing, ~50% of the cells still expressed the transduced genes as determined by hCD2 expression (data not shown). Cells were fixed in 1% formalin-buffered saline, permeabilized in 0.1% Triton X-100, and stained for TLP using affinity-purified anti-TLP antiserum plus goat anti-rabbit IgG-FITC (A to D). Infected cells were costained for actin using phalloidin-rhodamine (A and C). Stained cells were analyzed using a confocal microscope at 40× magnification. Similar results were obtained using cells transduced with TLP-B (data not shown).

CRP2, and MLP and possibly allowing it to form a different structure by differential chelation of the zinc ion.

By aligning the TLP-A and TLP-B cDNA sequences to the genomic DNA sequence, it was determined that the TLP gene spans a total of 2.8 kb with eight exons and seven introns. We deduced that TLP-A and TLP-B cDNAs were generated from alternatively spliced transcripts using two different splice donor sites in exon 7 (Fig. 2C).

TLP expression is thymus specific and is restricted to the stromal compartment. Based on Northern analysis of mRNA from several adult mouse tissues, TLP expression appeared to be thymus specific (Fig. 3A). Since there was one mouse EST from mammary tissue which matched the TLP sequence, we examined TLP expression in mammary tissue isolated from an 8-week-old lactating mouse and in mammary epithelial cell line C57 (Fig. 3B). By Northern analysis, we were unable to detect

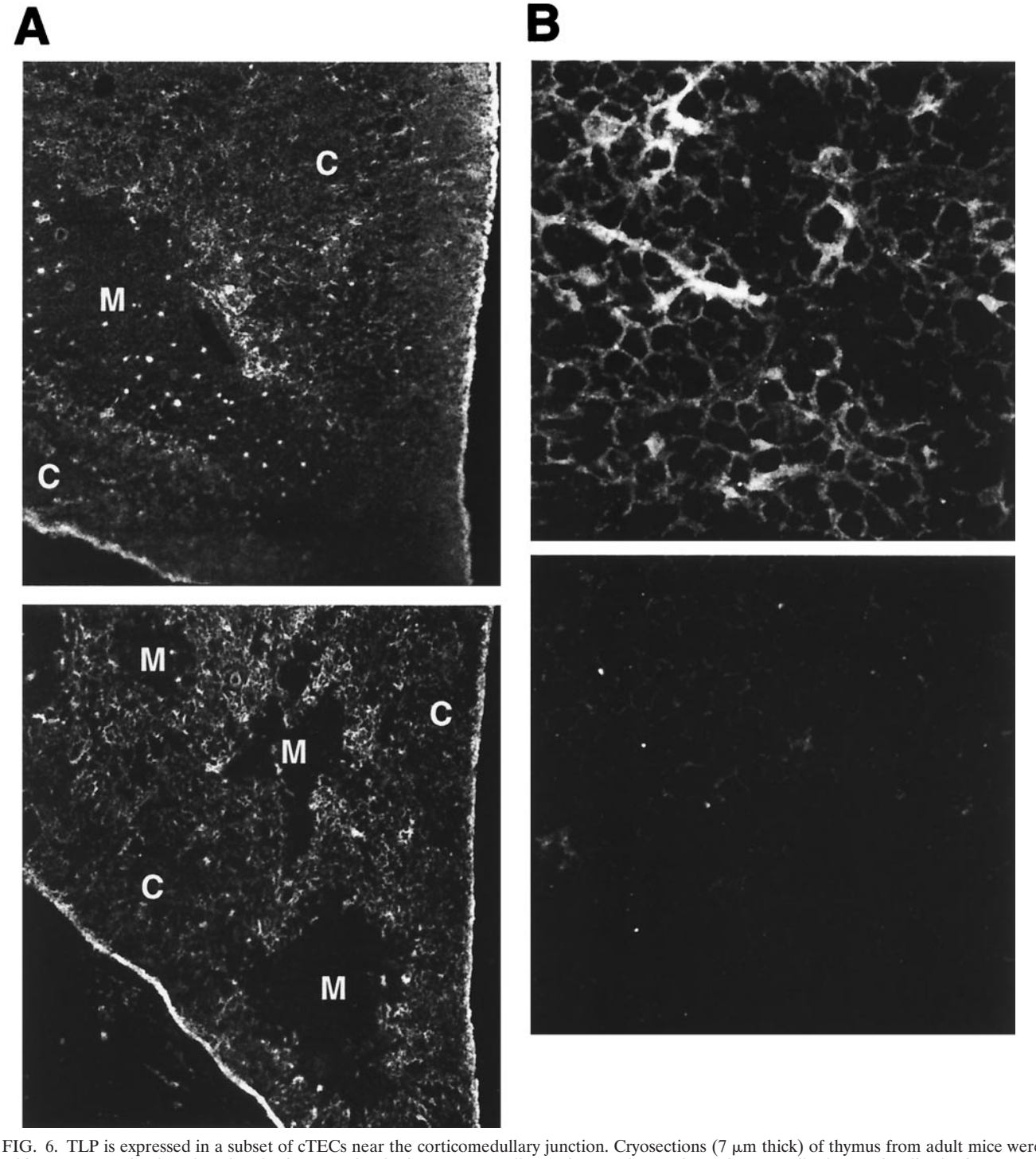


FIG. 6. TLP is expressed in a subset of cTECs near the corticomedullary junction. Cryosections (7 μm thick) of thymus from adult mice were fixed in acetone, stained, and analyzed using a confocal microscope. In all experiments shown, the anti-TLP antibody was visualized using Vector Red and Vecta Stain ABC-AP (Vector Laboratories). Cortical (C) and medullary (M) regions are indicated. Results shown are representative of several experiments. Large aggregates of fluorescence were sometimes observed in the medulla, even in sections from $TLP^{-/-}$ thymus (data not shown) and are a staining artifact. (A) C57BL/6 (top) and $Rag2^{-/-}$ OT-1 (bottom) mouse thymus sections were stained using affinity-purified anti-TLP antiserum (magnification, ×10). (B) Thymus sections from C57BL/6 mice were stained with affinity-purified anti-TLP antiserum (top) or rabbit IgG (bottom) (magnification, ×100). (C) Thymus sections from C57BL/6 mice were costained with affinity-purified anti-TLP antiserum and ERTR5 (top), 6C3 (middle), or NLDC145 (bottom) (magnification, ×36). ERTR5, 6C3, and NLDC145 were visualized with a FITC-conjugated goat anti-rat secondary Ab.

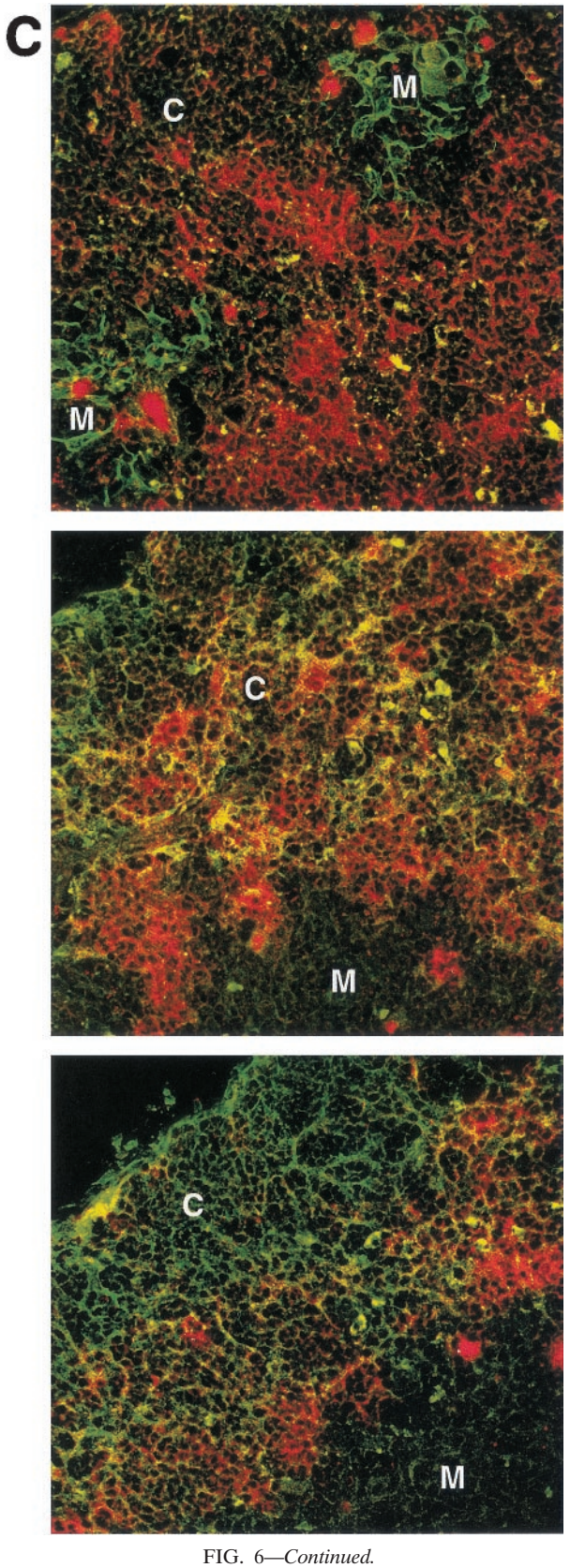


FIG. 6—*Continued.*

TLP message in either mammary tissue or the cell line, even after a long exposure.

To determine in which thymus compartment TLP was expressed, we separated thymocytes from stromal cells of a C57BL/6 mouse thymus and performed Northern analysis (Fig. 3C). CD4, expressed only in thymocytes, and MHC-II, expressed only in stromal cells, were probed as controls for cross contamination of RNA. TLP appeared to be expressed only in the stromal cells, as no TLP message was detected in thymocytes, even after a long exposure. We noted that TLP message was significantly less abundant than MHC-II, which is expressed in the majority of thymic epithelial cells. Based on this expression analysis, the TLP gene appeared to be a thymus-specific LIM gene whose expression is restricted to the stromal compartment.

TLP is a 23-kDa cytoplasmic protein which does not appear to associate with actin. To identify the TLP protein, we raised antibodies against a GST-TLP fusion protein containing TLP aa 78 to 115, a region between the two LIM domains (Fig. 2A). This sequence resides in both TLP isomers and occurs in a region with little similarity to corresponding regions of CRP1, CRP2, and MLP. The polyclonal antiserum was affinity purified and used for immunoblot analysis of cell lysates from COS cells, which do not express TLP, and COS cells transfected with a C-terminal myc-tagged version of TLP (TLP-myc) (Fig. 4). A specific band corresponding to an apparent molecular mass of 24 kDa was detected with the anti-TLP antiserum in transfected, but not untransfected, cells (Fig. 4A, left). This size corresponded to the predicted size of TLP plus the 10-aa myc tag. A band of this size was not detected using preimmune serum from the same rabbit (data not shown). In identical blots probed with anti-human c-myc Ab (anti-myc), a protein of the same size was detected only in transfected COS cells (Fig. 4A, right).

To identify endogenous TLP, we used the anti-TLP antibody in an immunoblot analysis of extracts from thymic stroma, thymocytes, and heart tissue (Fig. 4B). COS cells transduced with the C-terminal myc-tagged TLP were used as a positive control. We detected a specific band for TLP only in the thymic stromal cells and the positive control. The apparent molecular mass of TLP in thymic stroma was 23 kDa, closely matching the predicted size. CRP1, CRP2, and MLP are expressed in heart (34). The absence of TLP in thymocytes and heart tissue correlated with the expression of TLP message and demonstrated the specificity of the affinity-purified antibody for TLP.

Among a panel of TEC lines (427.1, 1308.1, 6.1.7, Z210R.1, 100.4, and TE71), we were unable to identify any which endogenously expressed TLP, even after treatment with cytokines which induce upregulation of MHC-II (data not shown). To determine the subcellular localization of TLP, we performed immunofluorescence confocal microscopy using TEC lines which had been transduced with TLP (Fig. 5). The cells were costained for TLP and actin. In both cortical (427.1) and medullary (Z210R.1) TEC lines, TLP localized to the cytoplasm. This was also true in COS cells transduced with TLP (data not shown). In contrast to what has been observed for CRP1, CRP2, and MLP, TLP did not appear to be strongly associated with actin, nor was there any evidence of nuclear localization. Thus, in epithelial cell lines, TLP is a cytoplasmic protein of approximately 23 kDa.

TLP is expressed in a subset of thymic cortical epithelial cells near the corticomedullary junction. To determine in which cells of the thymic stromal compartment TLP was expressed, we performed immunofluorescence confocal microscopy of thymus sections from a C57BL/6 mouse (Fig. 6). At 10× magnification, we observed scattered TLP expression in the outer cortex of the thymus and more-abundant expression of TLP in the deep cortex, especially near the corticomedullary junction (Fig. 6A, top). No expression of TLP was observed in the medulla (the large aggregates of fluorescence are due to a staining artifact). The thymuses of RAG2$^{-/-}$ OT-1 mice appear to have a disorganized medullas, presumably due to the higher-than-normal degree of selection occurring in these thymuses (12, 37). In thymus sections from these mice there was some TLP expression in the outer cortex but increased expression near each corticomedullary junction (Fig. 6A, bottom). In contrast, there was little or no staining in the RAG2$^{-/-}$ TAP1$^{-/-}$ OT-1 thymus, which does not have a medulla (data not shown).

At 100× magnification of C57BL/6 mouse thymus sections, it appeared that TLP was expressed in the stromal cells between the densely packed thymocytes (Fig. 6B, top). No staining was observed when rabbit IgG was used as the primary Ab (Fig. 6B, bottom) or when the secondary Ab was eliminated (data not shown).

To assess whether TLP expression occurred in a previously defined subpopulation of thymic stromal cells, we performed experiments in which we costained for TLP together with markers of known subsets of thymic stroma (Fig. 6C). There was no overlap between TLP and ERTR5 (75) (specific for medullary epithelial cells; Fig. 6C, top). 6C3 stains a subset of thymic cortical epithelial cells (1). NLDC145 stains thymic DCs and a subset of cortical epithelial cells (42). There was some overlap of TLP staining with each of these two markers (Fig. 6C, middle and bottom, respectively), confirming that TLP is expressed in cortical epithelial cells. However, there were some cells which expressed TLP but were not positive for NLDC145 or 6C3. This was especially true for cells near the corticomedullary junction. Thus, TLP appears to be a novel marker for a subpopulation of cTECs which occur most frequently, although not exclusively, near the corticomedullary junction.

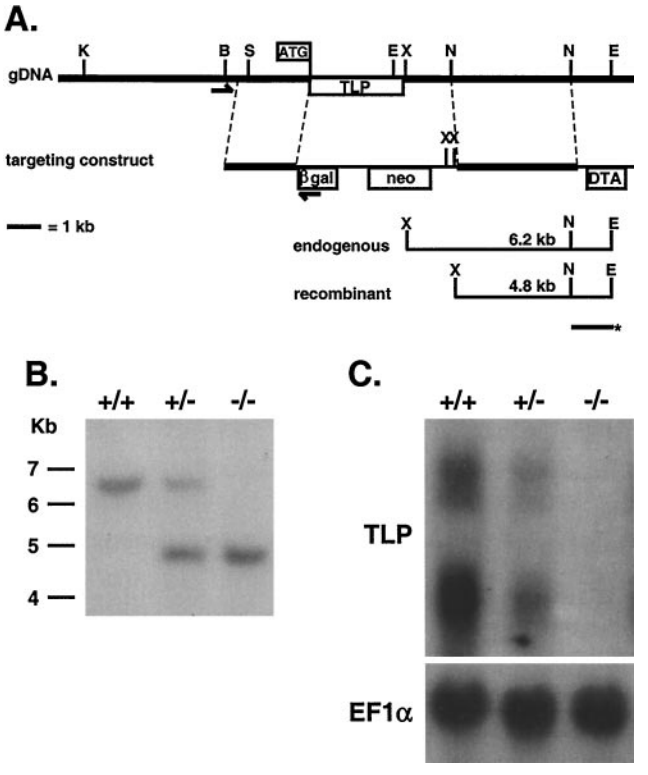
Targeted disruption of TLP results in a reduction of thymus cellularity. To determine whether TLP was required for T-cell development, we generated mice with a targeted disruption of the *Tlp* locus (Fig. 7). The progeny of TLP$^{+/-}$ intercrosses exhibited a normal Mendelian ratio of TLP$^{+/+}$, TLP$^{+/-}$, and TLP$^{-/-}$ mice, indicating that TLP is not required for embryonic development (data not shown). The TLP$^{-/-}$ mice produced no TLP message in the thymus (Fig. 7C) or protein that could be detected by immunohistochemistry (Fig. 7D). Despite this, T-cell development and thymus architecture in the TLP$^{-/-}$ mice appeared to be normal compared to those in the TLP$^{+/+}$ littermate controls. There was no difference between TLP$^{-/-}$ and TLP$^{+/+}$ mice in the percentages of CD4SP, CD8SP, DP, and DN subpopulations (Fig. 8A) or in the levels of surface expression of TCR$\alpha\beta$, TCR$\gamma\delta$, CD24, CD5, and CD69 on each thymocyte subpopulation (data not shown). Within the DN subpopulation, there was no significant difference in the percentages of CD44$^+$ CD25$^-$, CD44$^+$ CD25$^+$, CD44$^-$ CD25$^+$, and CD44$^-$ CD25$^-$ subpopulations (data not shown). In addition, the TLP$^{-/-}$ and TLP$^{+/+}$ mice had similar percentages of peripheral CD4 and CD8 T cells. To determine whether positive or negative selection of a limited TCR repertoire might be affected by the absence of TLP, we bred the TLP$^{-/-}$ mice to mice expressing one of various transgenic TCRs: OT-1 (14), TCli (81), or HY (38) (data not shown). The OT-1 and HY transgenic TCRs are MHC-I restricted, and the TCli transgenic TCR is MHC-II restricted. In mice bearing any one of the transgenic TCRs, there was no difference in the degree of positive selection of thymocytes between TLP$^{-/-}$ mice and TLP$^{+/+}$ littermate controls. Likewise, in HY males there was no difference in negative selection of thymocytes between TLP$^{-/-}$ mice and TLP$^{+/+}$ littermate controls. Taken together, these data indicate that TLP is not required for either positive or negative selection of T cells.

Although T-cell selection was apparently unaffected by the absence of TLP, there was a reduction in thymus size for TLP$^{-/-}$ mice compared to that for either the TLP$^{+/-}$ or TLP$^{+/+}$ littermate controls. On average, the TLP$^{-/-}$ mice had 30% fewer thymocytes than the controls (Fig. 8B). This decrease was observed in TLP$^{-/-}$ mice on either a mixed C57BL/6 and 129Sv background or a pure 129Sv background. The difference in thymic cellularity between TLP$^{-/-}$ mice and littermate controls was consistent over a wide range of ages, from 3 to 26 weeks old. Since the percentages of each thymocyte subpopulation were the same for TLP$^{-/-}$ and littermate controls, there was an equivalent percent decrease in cellularity in every subpopulation. Despite the lower number of thymocytes, there was no decrease in the number of spleen cells in TLP$^{-/-}$ mice (Fig. 8B).

DISCUSSION

We cloned a novel LIM-only gene which we named the *Tlp* gene. TLP exists as two isomers, TLP-A and TLP-B, which are identical except for the C-terminal 19 or 20 aa. We do not know whether there is any functional difference between the two isomers. TLP appears to be a member of the CRP family of LIM-only proteins, based on the degree of similarity in protein sequence and structure. Like the CRPs, TLP has two LIM domains, each of which is followed by a glycine-rich region, a feature unique to the CRPs (18). The LIM domains in each of the CRPs have been shown to mediate specific protein-protein interactions including homodimerization, association with actin filaments, and interactions with proteins found at sites of focal adhesion (8, 27, 64). MLP also binds to MyoD, a basic helix-loop-helix protein critical for myogenesis (40). Such protein-protein interactions are thought to be important both for intracellular targeting and for the formation of multimeric protein complexes which regulate cellular differentiation (10). TLP is likely to engage in specific protein-protein interactions via one or both of its two LIM domains. Identification of putative protein binding partners will be useful in understanding the role of TLP in thymus development.

TLP is not the only LIM-only protein found in the hematopoietic system. LMO2, also composed primarily of two LIM domains, is expressed in erythroid precursor cells and is essential for erythroid development (77) and adult hematopoiesis (82). Defects in LMO2$^{-/-}$ mice are likely due to the loss of the



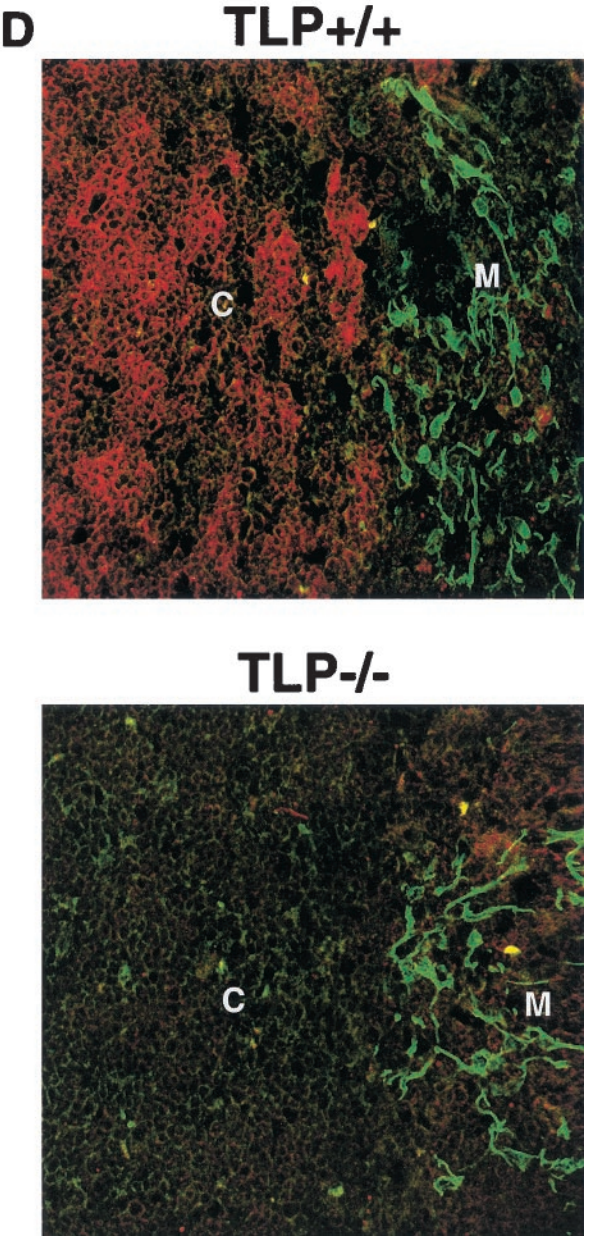


FIG. 7. Targeted disruption of the TLP locus. (A) Schematic diagram of the targeting construct used for generating TLP knockout mice. Homologous recombination between the targeting construct and the genomic TLP locus results in replacement of the entire TLP coding sequence (open box), except the start codon, with the β-Gal gene (βgal) and a gene for neomycin resistance (neo). In the recombinant, the TLP start codon is in frame with the β-Gal ORF. ES cells and mice with a recombinant allele were identified by PCR of the 5′ end using primers annealing as shown. DTA, diphtheria toxin A gene; *, probe for Southern analysis. (B) Southern analysis of genomic DNA from $TLP^{+/+}$, $TLP^{+/-}$, and $TLP^{-/-}$ mice. Recombination at the 3′ end was confirmed by Southern analysis of 10 μg of genomic DNA digested with *Xho*I and *Eco*RI, using as a probe an *Nhe*I-*Eco*RI fragment downstream of the recombinant region. (C) Northern analysis of 10 μg of whole-thymus total RNA from $TLP^{+/+}$, $TLP^{+/-}$, and $TLP^{-/-}$ mice using a probe for TLP and EF1α. (D) Immunofluorescence confocal microscopy of thymus sections from $TLP^{+/+}$ and $TLP^{-/-}$ mice. Cryosections (7 μm thick) were costained with affinity-purified anti-TLP antiserum and ERTR5 and analyzed as for Fig. 6C (magnification, ×36). Cortical (C) and medullary (M) regions are indicated.

interactions of LMO2 with TAL1, a basic helix-loop-helix protein which is also critical for erythropoiesis (72). CRIP, a LIM-only protein with a single LIM domain, is expressed in rats primarily in intestine but also in immune system cells, including peritoneal macrophages and peripheral blood mononuclear cells, and in the thymus and spleen. Although expression of CRIP has not been detected in hematopoietic cells and tissues of mice, overexpression of CRIP under the control of the rat CRIP promoter in mice resulted in increased expression in the intestine, thymus, spleen, and lung, apparently causing a 50% decrease in circulating lymphocytes (17). TLP is, however, the first identified LIM-only protein whose expression in the immune system is thymus specific and restricted to the stromal compartment.

We characterized the structure of the *Tlp* gene in mice and found the gene to consist of eight exons and seven introns, spanning about 2.8 kb. By aligning the TLP-A and TLP-B cDNAs to the genomic sequence, we were able to determine that they are generated by alternative splicing from two different splice donor sites in exon 7 to exon 8. By searching the human genome sequence, we identified a human homologue for the *Tlp* gene. The human *Tlp* gene homologue mapped to chromosome 6 and appeared to have a structure similar to that of the *Tlp* gene in mice. Based on the high degree of sequence identity, it appears that TLP is evolutionarily conserved.

We characterized the expression of TLP by Northern analysis and immunohistochemistry. TLP was isolated on the basis of higher expression in a selecting thymus than in a nonselecting thymus. Among a panel of adult tissues, we detected TLP only in the thymus, and, within the thymus, specifically in the

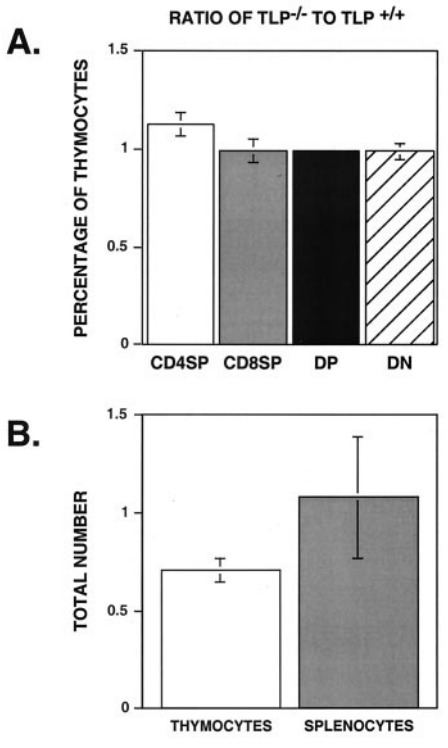


FIG. 8. Targeted disruption of the TLP gene results in a decrease in thymic cellularity. Thymocytes and splenocytes from eight pairs of $TLP^{-/-}$ and $TLP^{+/+}$ littermates (gender matched; from 3 to 26 weeks old) were counted and analyzed by flow cytometry. (A) Averages of the percentages of four thymocyte subpopulations (CD4SP, CD8SP, DP, and DN) expressed as ratios of subpopulations in $TLP^{-/-}$ mice to those in $TLP^{+/+}$ mice. Error bars are standard errors of the means (SEM). The *P* values are, respectively, 0.040, 0.470, 0.110, and 0.390 by the Student *t* test. (B) Cellularity expressed as a ratio of the number of thymocytes or splenocytes in $TLP^{-/-}$ mice to the number in $TLP^{+/+}$ mice. Error bars are SEM. The *P* values are, respectively, 0.007 and 0.270 by the Student *t* test.

stromal cell compartment. Despite matching an EST from mouse mammary tissue, we were unable to detect TLP expression in either mammary tissue or a mammary epithelial cell line. This apparent discrepancy could be due to the fact that the mammary-tissue source of the EST sequence was 4-week-old (prepubertal) female mice, while our RNA sample was from an 8-week-old lactating mouse. Alternatively, expression of TLP in mammary tissue may simply be too low to detect by Northern analysis. $TLP^{-/-}$ females were fully capable of nursing their pups (data not shown). By immunohistochemistry, TLP was expressed within the thymus in a subset of cTECs and was most abundantly, although not exclusively, expressed near the corticomedullary junction. This pattern of expression in the thymus is unique. It was of particular interest because expression in microenvironments in the cortex, especially near the corticomedullary junction, correlated with a potential role for TLP in selection of thymocytes.

We identified the TLP protein as a 23-kDa protein which localizes to the cytoplasm in both mTEC and cTEC lines. By immunohistochemical studies, we did not observe a strong association of TLP with actin filaments. This differs from what has been observed for CRP1, CRP2, and MLP in colocalization studies (7, 8, 45) and suggests that TLP has a function distinct from that of the other family members. Interestingly, we were unable to identify any epithelial cell line with endogenous TLP expression. It is known that expression of other genes in TECs is dependent on close contact with thymocytes. For example, MHC-II expression decreases dramatically within 24 h after removal from the thymus but can be induced by the addition of cytokines. TLP appeared to be scattered throughout the cortex of the thymus in several foci of expressing cells, suggesting that the microenvironment may influence TLP expression. The same cytokines that induce MHC-II, however, did not induce expression of TLP (data not shown).

Based on the thymus-specific expression of TLP, its upregulation in a selecting thymus compared to its expression in a nonselecting thymus, its enriched expression in cTECs near the corticomedullary junction, and the homology of the associated gene to a family of genes (LIM genes) involved in development, we hypothesized that TLP was likely to have an important role in T-cell development. To determine whether this was the case, we generated mice with a targeted disruption of the *Tlp* locus and analyzed the effect of the absence of TLP on T-cell development. The $TLP^{-/-}$ mice had no detectable message or protein. Despite extensive analysis of T-cell subpopulations and markers of development, we were unable to identify any defect in T-cell selection in the $TLP^{-/-}$ mice compared to littermate controls, in either adults or day 1 neonates. This was true even when we analyzed the effect of the knockout in mice with a limited TCR repertoire (TCR transgenic mice). In addition, we observed no symptoms of autoimmunity, even in older mice (data not shown). Since the pattern of TLP expression correlated well with a putative role in thymocyte selection, it was surprising that there was no discernible effect on positive (or negative) selection of thymocytes in the absence of TLP. This could be interpreted to mean that TLP has no role in T-cell development. Alternatively, there may be redundant proteins expressed in cTECs which were able to compensate for the loss of TLP. Although there have been reports of low CRP1, CRP2, and MLP expression in the thymus, it is unlikely that any of these proteins would be able to substitute for TLP. Each CRP is only about 30% identical to TLP. In addition, TLP does not exhibit the strong association with actin that each of the CRPs does, suggesting a distinct role for TLP. Although TLP was upregulated in an MHC-I-restricted selecting thymus ($Rag2^{-/-}$ OT-1) compared to its expression in a nonselecting ($Rag2^{-/-}$ $Tap1^{-/-}$ OT-1) thymus, we did not observe this same hierarchy of expression in MHC-II-restricted selecting and nonselecting thymuses ($Rag2^{-/-}$ TCli and $Rag2^{-/-}$ $Ii^{-/-}$ TCli; data not shown). In addition, we observed significant TLP expression in $RAG^{-/-}$ and $TCR\alpha^{-/-}$ thymuses (by Northern analysis; data not shown), suggesting that regulation of TLP expression may be complex and not necessarily correlated with the degree of selection in all systems. The decreased expression in $Rag2^{-/-}$ $TAP^{-/-}$ OT-1 mice compared to that in $Rag2^{-/-}$ OT-1 mice is not likely to be due solely to the absence of MHC-I expression or TAP function per se, since there was no decrease in TLP expression in $TAP^{-/-}$ and $\beta 2m^{-/-}$ thymuses compared to that in a C57BL/6 mouse thymus by Northern analysis (data not shown).

Although there was no apparent effect of targeted disruption of *Tlp* on T-cell development, there was a significant decrease

(30% ± 0.06%; $n = 8$, $P = 0.007$) in thymic cellularity in the $TLP^{-/-}$ mice compared to that in either $TLP^{+/-}$ or $TLP^{+/+}$ littermate controls. Despite a decrease in the message level of TLP in $TLP^{+/-}$ mice, there was no difference in cellularity between $TLP^{+/-}$ and $TLP^{+/+}$ mice (data not shown), suggesting that lower levels of TLP were sufficient to achieve normal cellularity. The decrease in cellularity in $TLP^{-/-}$ thymuses occurred equally in all thymic subpopulations and over a wide range of ages (from 3 to 26 weeks), possibly reflecting a role for TLP in thymus organogenesis. It did not appear to be the result of increased emigration since the numbers of splenocytes in $TLP^{+/-}$ mice and $TLP^{+/+}$ littermate controls were the same.

Although we do not know by what mechanism deletion of TLP results in decreased thymocyte cellularity, it is worth speculating what function TLP may have in thymus development. Given the interdependent nature of thymocyte and stromal cell development and the apparently normal architecture of the $TLP^{-/-}$ thymus, it is reasonable to assume that there was a similar decrease in the number of stromal cells in $TLP^{-/-}$ thymuses. Decreased stromal cellularity or a defect in normal cTEC development or differentiation may be the direct effect of the TLP knockout, which could, in turn, dictate the number of thymocytes which could enter or occupy the thymus. Thus, although TLP is not required for selection of T cells, it appears to have a role in normal thymus development.

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