Genomic Characterization of LIGHT Reveals Linkage to an Immune Response Locus on Chromosome 19p13.3 and Distinct Isoforms Generated by Alternate Splicing or Proteolysis

Steve W. Granger, Kris D. Butrovich, Pantea Houshmand, Wilson R. Edwards and Carl F. Ware

*J Immunol* 2001; 167:5122-5128; doi: 10.4049/jimmunol.167.9.5122

http://www.jimmunol.org/content/167/9/5122

<table>
<thead>
<tr>
<th>References</th>
<th>This article cites 34 articles, 16 of which you can access for free at: <a href="http://www.jimmunol.org/content/167/9/5122.full#ref-list-1">http://www.jimmunol.org/content/167/9/5122.full#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Subscription</td>
<td>Information about subscribing to <em>The Journal of Immunology</em> is online at: <a href="http://jimmunol.org/subscription">http://jimmunol.org/subscription</a></td>
</tr>
<tr>
<td>Permissions</td>
<td>Submit copyright permission requests at: <a href="http://www.aai.org/About/Publications/JI/copyright.html">http://www.aai.org/About/Publications/JI/copyright.html</a></td>
</tr>
<tr>
<td>Email Alerts</td>
<td>Receive free email-alerts when new articles cite this article. Sign up at: <a href="http://jimmunol.org/alerts">http://jimmunol.org/alerts</a></td>
</tr>
</tbody>
</table>
Genomic Characterization of LIGHT Reveals Linkage to an Immune Response Locus on Chromosome 19p13.3 and Distinct Isoforms Generated by Alternate Splicing or Proteolysis\textsuperscript{1,2}

Steve W. Granger, Kris D. Butrovich, Pantea Houshmand, Wilson R. Edwards, and Carl F. Ware\textsuperscript{3}

LIGHT is a member of the TNF cytokine superfamily that signals through the lymphotoxin (LT)\textalpha and LT\textbeta (1). LIGHT is a type II transmembrane glycoprotein that is transiently expressed on the surface of activated T lymphocytes (1) and dendritic cells (2). Two differentially expressed cell surface receptors mediate LIGHT signaling, the herpesvirus entry mediator (HVEM also known as HveA), prominent on T cells, and the LT\textbeta R, found on stromal cells, but absent on lymphocytes (1). LIGHT interacts with a soluble binding protein, decoy receptor 3, which also interacts with Fas ligand (FasL) (3, 4).

The interaction of LIGHT with the LT\textbeta R induces proinflammatory gene expression through activation of the transcription factor NF-kB, similar to the LT\textalpha complex, the ligand required for lymphoid organogenesis and development of NK (5) and NK-T cells (6). LIGHT induces apoptosis in susceptible colon carcinoma cells via the LT\textbeta R (7), and in vivo, transduction of tumor cells with LIGHT cDNA suppresses tumor outgrowth (8). Evidence is accumulating that LIGHT signaling through HVEM may function as a costimulatory molecule for T cells, including the enhancement of T cell proliferation and secretion of IFN-\gamma (2, 9, 10). Additionally, inhibition of LIGHT with soluble LT\beta R-Fc decoy or anti-LIGHT Ab suppresses graft vs host disease (10) indicating LIGHT is involved in effector functions mediated by T cells. LIGHT may also function as a direct viral deterrent. HSV infection induces premature death of activated T cells (11) and can block maturation of dendritic cells (12, 13) potentially leading to localized immune suppression. Envelope glycoprotein D binds HVEM as one of the cellular entry routes used by HSV (14). Glycoprotein D directly competes for the binding of membrane LIGHT to HVEM, whereas LIGHT can interfere with HSV entry by down-regulation of HVEM (1, 15). These observations suggest that LIGHT may be an integral part of host immunity to herpesvirus, which is strongly supported by recent observations on the role that LIGHT and LT\alpha\beta play in resistance to CMV (16). The interaction of viral gene products with TNF superfamily (TNFSF) members may have shaped the evolutionary course of these molecules, which prompted us to examine the genetic organization of LIGHT (TNFSF14) for insights into the regulation of this cytokine.

Here, we define the molecular genetics of the human LIGHT genomic locus, revealing close linkage to CD27 ligand and 4-1BB ligand, and the third complement protein (C3), which positions LIGHT within the MHC paralog on chromosome 19p13.3. An alternately spliced isoform of LIGHT mRNA that encodes a transmembrane-deleted form is detected in activated T cells and gives rise to a nonglycosylated protein that resides in the cytosol. Furthermore, membrane LIGHT is shed from the cell surface of human 293 T cells. These studies reveal new mechanisms involved in regulating the physical forms and cellular compartmentalization of LIGHT that may contribute to the regulation and biological function of this cytokine. The Journal of Immunology, 2001, 167: 5122–5128.

\textsuperscript{1} This work was supported in part by U.S. Public Health Service, National Institutes of Health Grants CA69381, AI03568, and A148073.
\textsuperscript{2} This is publication 428 from The La Jolla Institute for Allergy and Immunology.

Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92121

Received for publication June 29, 2001. Accepted for publication August 24, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Abbreviations used in this paper: LT, lymphotoxin; BAC, bacterial artificial chromosome; FasL, Fas ligand; HVEM, herpesvirus entry mediator; TNFSF, TNF superfamily; NP40, Nonidet P-40; Chr, chromosome; PNGase F, peptide-N-glycosidase F; CD27, CD27 ligand; 4-1BBL, 4-1BB ligand; C3, third complement protein; endo H, endoglycosidase H.

Copyright © 2001 by The American Association of Immunologists

0022-1767/01/$02.00

Materials and Methods

Cells, cytokines, and Abs

The human II-23 cell line (D7 subclone), a CD4\textsuperscript{+} T cell hybridoma line (17), was maintained in RPMI 1640 containing 10% FBS (HyClone Laboratories, Logan, UT) and 100 U/ml penicillin/100 \mu g/ml streptomycin (Life Technologies, Grand Island, NY). II-23 cells were activated with 100 ng/ml PMA or PMA and 1 \mu g/ml ionomycin for 4 h. Human kidney 293
cells expressing the adenovirus large T Ag (293T) (American Type Culture Collection, Manassas, VA) were grown in DMEM containing 10% FBS and antibiotics. Human PBL were isolated by Ficoll gradient centrifugation and adherent cell depletion as described previously (1). B lymphocytes were removed by passage through nylon wool, and the T lymphocytes were activated with anti-CD3 (OKT3) (1 μg/ml) and anti-CD28 (1 μg/ml) (BD Biosciences, Mountain View, CA) and cultured in RPMI 1640/FBS with IL-2 (10 ng/ml). After 7 days of culture, these T cells were activated with PMA (100 ng/ml) and ionomycin (1 μg/ml) in fresh medium at 2 × 10^6 cells/ml as described previously (1). Rat anti-human LIGHT was prepared by immunization with 50 μg of purified LIGHT66, a recombinant truncated form with a deletion of the cytoplasmic and transmembrane regions generating a soluble protein as previously described (7). Anti-human LIGHT Omniclone is a bacterially expressed combinatorial Ab containing V_H and V_e-chains generated from a BALB/c mouse immunized with recombinant soluble human LIGHT66 (Bioptics Diagnostics, San Diego, CA) (18). This Ab reacts with natural and recombinant human LIGHT in flow cytometry and immunoprecipitation; no cross-reactivity was observed with TNF, LTαβ, or mouse LIGHT (a detailed description of the properties of this reagent is in preparation). Mouse anti-methamphetamine Omniclone was used as an isotype control and was provided by G. Valkirs (Biosite Diagnostics, San Diego, CA) (18). This Ab reacts with natural and recombinant human LIGHT in soluble human LIGHTt66 (Biosite Diagnostics, San Diego, CA) (18). This analysis was performed using Superscript II (Life Technologies) as described previously (21). PCR amplification, using oligonucleotide primers derived from the human LIGHT cDNA sequence (forward 5'-TGACCTGTGTTG GTTGGATGGA-3', reverse 5'-CCCTCTCCACACCATAAGGC-3') was accomplished using the following amplification schedule: 95°C for 2 min; 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; and 72°C for 10 min. Following amplification, products were analyzed by agarose (1.5%) gel electrophoresis. The gels were stained using 1 μg/ml ethidium bromide and photographed under UV trans-illumination with an alpha imager (Alpha Innotech, San Leandro, CA).

**RT-PCR analysis**

RNA was isolated from 2 × 10^6 cells using 1 ml of TRizol (Life Technologies) following the manufacturer’s protocol. First-strand cDNA synthesis was performed using Superscript II (Life Technologies) as described previously (21). PCR amplification, using oligonucleotide primers derived from the human LIGHT cDNA sequence (forward 5'-TGACCTGTGTTG GTTGGATGGA-3', reverse 5'-CCCTCTCCACACCATAAGGC-3') was accomplished using the following amplification schedule: 95°C for 2 min; 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; and 72°C for 10 min. Following amplification, products were analyzed by agarose (1.5%) gel electrophoresis. The gels were stained using 1 μg/ml ethidium bromide and photographed under UV trans-illumination with an alpha imager (Alpha Innotech, San Leandro, CA).

**Transfections**

Human embryonic kidney 293T cells, in six-well dishes (50% confluent), were transfected with 3 μg of CD27L cDNA per well, using the calcium phosphate coprecipitation method (22). The cells were incubated with the precipitate for 12 h, and then fresh complete medium was added for 48 h to achieve maximal protein expression.

**Receptor-mediated ligand precipitation and Western blot analysis**

Tissue culture supernatants were harvested from transfected 293T cells, and debris was removed by centrifugation at 1000 × g for 5 min. Supernatants were then treated with a protease inhibitor mixture (1 mM PMSF, 10 mM iodoacetamide, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.02% azide) and adjusted to a final concentration of 1% Nonidet P-40 (NP40). Cellular extracts were prepared by treatment of cell pellets (2.5 × 10^6 cells) with 0.7 ml of 1% NP40 lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM EDTA, 1 mM PMSF, 10 mM iodoacetamide, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.02% azide. Supernatants or detergent lysates were precleared by incubation with 10 μg/ml human IgG or mouse isotype control and 30 μl of protein G-Sepharose beads (Pharmacia, Peapack, NJ) for 2 h at 4°C with rotation. Ligands were precipitated from supernatants or lysates by incubation with 10 μg/ml HVEM-Fc, 10 μg/ml TNF-R1-Fc, or 10 μg/ml mouse anti-LIGHT Omniclonal Ab for 2 h at 4°C with protein G-Sepharose beads. Washed immunoprecipitates were treated with recombinant forms of endoglycosidase H (endo H) and peptide-N-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) for 1 h at 37°C. Samples were resolved using a Tris-glycine SDS-polyacrylamide (14%) gel (NOVEX, San Diego, CA). Tissue culture supernatants were harvested from transfected 293T cells, and debris was removed by centrifugation at 1000 × g for 5 min. Supernatants were then treated with a protease inhibitor mixture (1 mM PMSF, 10 mM iodoacetamide, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.02% azide) and adjusted to a final concentration of 1% Nonidet P-40 (NP40). Cellular extracts were prepared by treatment of cell pellets (2.5 × 10^6 cells) with 0.7 ml of 1% NP40 lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM EDTA, 1 mM PMSF, 10 mM iodoacetamide, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.02% azide. Supernatants or detergent lysates were precleared by incubation with 10 μg/ml human IgG or mouse isotype control and 30 μl of protein G-Sepharose beads (Pharmacia, Peapack, NJ) for 2 h at 4°C with protein G-Sepharose beads. Washed immunoprecipitates were treated with recombinant forms of endoglycosidase H (endo H) and peptide-N-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) for 1 h at 37°C. Samples were resolved using a Tris-glycine SDS-polyacrylamide (14%) gel (NOVEX, San Diego, CA), and proteins were transferred to Polyscreen polyvinylidene difluoride transfer membrane (NEN, Boston, MA) using a semidry blotting unit (Fisher, Pittsburgh, PA). Immune complexes were detected with goat F(ab')2 anti-rat IgG conjugated to HRP and detected by ECL (Pierce SuperSignal; Pierce, Rockford, IL) as described (7).

**FIGURE 1.** A, Chromosomal localization of human LIGHT and the costimulatory locus. The chromosomal location of LIGHT was determined by fluorescence in situ hybridization (FISH) of metaphase chromosomes using hybridization probes including the entire BAC clone containing the LIGHT genomic locus and Chr19-specific DNA. Cohybridization of each probe, indicated by arrows, on a single chromosome indicated that LIGHT is located at the terminus of the short arm of Chr19. B, Diagram of the 19p13.3 region. C, Organization of the C3, LIGHT, CD27L, 4-1BBL immune response locus. Arrows indicate the transcriptional orientation of each gene, and solid blocks represent exons. LIGHT is separated from C3 by 7.78 kb, from CD27L by ~79 kb, whereas CD27L and 4-1BBL are separated by ~235 kb. D, Organization of the FasL, GITR ligand, and OX40 ligand genomic locus. FasL is separated from GITR by 374 kb. GITR and OX40L are separated by 134 kb. The accession numbers of BAC clones or assembled contigs that contain the various loci are shown above each diagram. E, Phylogenetic analysis of various TNFSF members. Sequences of the extracellular domains were aligned using ClustalW (BLOSUM series matrix), and phylogeny was inferred using the Neighbor joining method and bootstrapped (1000 reps) (MacVector 7.0). The tree is unrooted and numbers indicate percentages of 1000 bootstrap replicates.
Cloning, sequencing, and sequence analysis

The human LIGHT locus-containing P1 clone was isolated by PCR screening a P1 library using the following LIGHT specific oligonucleotide primers: forward 5'-GCTCTCGAGGAGCAGCTGATACAA-3' and reverse 5'-TGGGTGAACCTGGAAGACCTTCCG-3' (IncyteGenomics, Palo Alto, CA). The LIGHT genomic locus was sequenced in both 5' and 3' directions using primers specific for the LIGHT cDNA sequence and cycle sequencing chemistry (Applied Biosystems, Foster City, CA). Sequences were analyzed on an Applied Biosystems prism 310 sequencer and contigs were assembled using the AssemblyLIGN program (MacVector 7.0; Oxford Molecular, Madison, WI). RT-PCR products were cloned using the TA cloning system (Invitrogen, San Diego, CA). The PCR amplicon was digested and ligated into the pCMV2 mammalian expression vector in frame with amino-terminal FLAG coding sequences or pCDNA3.1++. Bacterial artificial chromosome (BAC) DNA was analyzed for gene content using the gene finding algorithm GenScan provided by the Massachusetts Institute of Technology Computational Biology Department (http://genes.mit.edu/GENSCAN.html) (23). Hypothetical proteins were identified by pBLAST search of GenBank. Consensus motifs for sequence-specific DNA binding proteins were identified using the transcription element search system (TESS) at the University of Pennsylvania (Philadelphia, PA) to search the TRANSFAC database (http://www.cbil.upenn.edu/tess/) (24).

Results and Discussion

The chromosomal location of LIGHT

In a BLAST search of the unfinished high throughput genomic sequence database, two BAC sequences (AC008760 and AC025343) from human Chr19 were identified; each contained the entire LIGHT gene (TNFSF14). The identity between LIGHT mRNA nucleotide sequences and the Chr19 genomic sequences, with the exception of introns, was absolute (E value of 0.0). This result was puzzling because a prior report assigned LIGHT to Chr16 by in situ hybridization of metaphase chromosomes (8). This discrepancy was resolved by screening a P1 phage library for the LIGHT genomic locus, and the entire P1 DNA clone was used for in situ hybridization of metaphase chromosomes (Fig. 1A). In this analysis, a Chr19 specific probe cohybridized with the P1 LIGHT probe and verified that the chromosomal location of LIGHT is 19p13.3.

The LIGHT gene resides adjacent to C3 and within a T cell costimulatory locus containing CD27L and 4-1BB.

Identification of the LIGHT-containing BAC clone AC008760 yielded 200 kb of genomic DNA for analysis of closely linked genes. Sequence analysis of this BAC clone revealed tight linkage of the C3 to LIGHT separated by only 7.78 kb with the 3' end of C3 adjacent to the 5' end of LIGHT (Fig. 1C). Several TNF family members localize in clusters, prompting a screen for related ligands in this region using the GenScan gene finding algorithm. The genomic locus for CD27L (CD70/TNFSF7) was identified on both BAC clones AC008760 and AC025343 and was mapped on NT 011098 to within 79 kb of LIGHT. In addition, the genomic locus of 4-1BBL (TNFSF9) was identified on a BAC clone (AC010503) that overlaps BAC AC025343 containing the LIGHT and CD27L genes. 4-1BBL was mapped on NT 011169 to within 235 kb of CD27L. Therefore, C3, LIGHT, CD27L, and 4-1BB-L all reside on 19p13.3 in a region that spans ~370 kb (Fig. 1, A and B).

The close genetic linkage of LIGHT to CD27L and 4-1BBL supports the possibility that this gene cluster arose from localized gene duplication events (25).

The positioning on Chr19p13.3 places LIGHT within a large genetic region paralogous to the MHC on Chr6p21.3, which is ~8 Mb in size (26, 27). The Chr19p13.1-1p13.3 MHC paralog is one of four regions thought to have arisen by chromosomal duplication, which include Chr1q21-2q5/p11-p32 and Chr9q33-3q4. Other TNF-related superfamily members map to these MHC paralogs, FasL and OX40 ligand to Chr1, and CD30 ligand and vascular endothelial growth inhibitor to Chr9. The evolutionary relationship of these paralogs has been the subject of much interest and controversy in understanding the origins of the MHC (28). These paralogs contain class I, complement and TNF-related genes among other conserved markers. The LIGHT locus is notably reminiscent of the TNF locus containing LTβ, TNF, and LTA closely linked to C2 and C4 within the MHC (29, 30). It should be noted that within the complement protein family, C3 displays the highest sequence similarity to C4 (31). In addition, LIGHT and LTβ share functionality and the highest sequence similarity within the TNF super family and LTβ resides near the C4 gene. During evolution, gene duplication events are often followed by translocations that either relocate the gene cluster to different chromosomes or break up the cluster altogether. Therefore, it is tempting to speculate that a translocation of the TNF-LT locus gave rise to the entire LIGHT

![Figure 2](http://www.jimmunol.org/Downloaded)

**FIGURE 2.** Identification of a novel LIGHT transcript (LIGHT ΔTM) expressed in activated II-23 cells and PBL. A, Diagram of LIGHT transcripts with the locations of the oligonucleotide primers used to amplify LIGHT cDNA by RT-PCR. B, LIGHT ΔTM expression in II-23 cells by RT-PCR analysis. RNA was isolated from II-23 cells 4 h postactivation. PCR products from the amplification of cDNA from untreated (lane 2) or PMA/ionomycin-treated (lane 3). II-23 cells were analyzed by agarose gel electrophoresis. As controls, RNA from II-23 cells activated with PMA and ionomycin without reverse transcriptase (lane 4) or water alone (lane 5). C, LIGHT ΔTM expression in human peripheral blood T lymphocytes by RT-PCR analysis. Anti-CD3-activated peripheral blood T cells were grown in IL-2 for 7 days and placed in fresh IL-2 medium (lane 2) or IL-2 medium with PMA and ionomycin for 2 h (lane 3) or 4.5 h (lane 4). At these time points RNA was extracted and analyzed for LIGHT expression by RT-PCR as in A above. As controls, RNA without reverse transcriptase from cells activated for 4.5 h (lane 5), water alone (lane 6), and 50 ng of LIGHT plasmid (lane 7) were used as templates for PCR.
locus or vice versa. However, CD27L and 4-1BBL have only three exons, whereas TNF, LTβ, LTα, and LIGHT have four exons suggesting that the LIGHT locus was not directly descended from the TNF/LT locus. This result compelled an examination of the genomic organization of TNF-related ligands on Chr1, where sufficient information on gene structure could be extracted. The gene content of the genomic DNA adjacent to the FasL gene, within the contig NT 000039, which is comprised of nine P1-derived artificial chromosome clones and two BAC clones of various sizes, contained FasL (TNFSF6), GITR ligand (TNFSF18), and OX40 ligand (TNFSF4) (Fig. 1D). Although the genes in the FasL locus span a greater distance than those of the LIGHT locus, the gene orientation and exon organization of FasL is strikingly similar to the LIGHT locus. Additionally, GITRL and OX40L contain three exons matching CD27L and 4-1BBL. Phylogenetic analysis of the amino acid sequence of the TNF family reveals significant sequence similarity in the proteins encoded by LIGHT and FasL loci that corresponds with their position in the gene clusters (Fig. 1E). (GITRL does not fit this tree well owing to significant divergence in sequence in the initial A β strand.) However, the sequence and organizational similarities do not apply when the LIGHT locus is compared with the LTα/β/TNF locus.

At a functional level, FasL and LIGHT exhibit significant amino acid sequence homology in their ectodomains (31%), second only to the similarity between LIGHT and LTβ (34%) (1). Although binding to distinct cellular receptors, FasL and LIGHT do share the ability to bind soluble DCR3 (4). Although various possible combinations of gene duplication and translocation may explain the origin of each gene cluster, collectively, the relationship between the FasL and LIGHT loci is suggestive of a duplicative event involving the entire gene cluster, presumably occurring during a chromosomal duplication. This result would support the idea that Chr19 and Chr1 MHC paralogs are more closely related to each other than the Chr6 paralog. Given the close linkage of LIGHT and C3, which is considered the primordial gene of C5 and C4 based on both phylogenetic and functional arguments (31), LIGHT may be primordial to LTβ and FasL. The chromosomal position of the LIGHT locus is conserved in mice; however, insufficient phylogenetic analysis is available for most TNFSF members limiting further analysis.

The genomic organization of LIGHT

The LIGHT gene spans 5.1 kb (from AUG to stop codon) and is comprised of four exons with similar organization to FasL, LTβ, and other TNF family members (Table I). Exon 1 of LIGHT encodes the first 73 amino acids of the polypeptide, which comprises the entire cytoplasmic tail (aa 1–38), transmembrane domain (aa 38–58), and the beginning of the extracellular stalk region. The second and third exons encode amino acids 74–85 and 86–100, respectively, which make up the stalk region and the beginning of the trimerization domain. The fourth exon encodes the remainder of the trimerization domain, the receptor binding domain (amino acids 101–240), and includes a site for N-linked glycosylation (Asn165). Based on structural homology with LTα (32) and supportive evidence from modeling and biochemical analysis, the trimerization domain of LIGHT folds into an anti-parallel β sandwich; wherein the receptor-binding sites are formed from adjacent subunits (7).

The LIGHT transcript has been previously reported as 2.5 or 2.7 kb by Northern blot analysis, although two considerably smaller

Table I. Intron/exon arrangement of LIGHT

<table>
<thead>
<tr>
<th>Exon</th>
<th>5' End</th>
<th>3' End</th>
<th>Size (bp)</th>
<th>Encodes (aa)</th>
<th>Size (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>ATGG-</td>
<td>-GCCT</td>
<td>219 (267 from cap)</td>
<td>1–74</td>
<td>73</td>
</tr>
<tr>
<td>Intron 1</td>
<td>GTGA-</td>
<td>-TCAG</td>
<td>2401</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 2</td>
<td>GACG-</td>
<td>-CAAG</td>
<td>37</td>
<td>74–85</td>
<td>12</td>
</tr>
<tr>
<td>Intron 2</td>
<td>GTGA-</td>
<td>-CCAG</td>
<td>258</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 3</td>
<td>AGCG-</td>
<td>-ACAG</td>
<td>42</td>
<td>86–99</td>
<td>14</td>
</tr>
<tr>
<td>Intron 3</td>
<td>GTGA-</td>
<td>-ACAG</td>
<td>1762</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 4</td>
<td>GGGC-</td>
<td>-AGG stop</td>
<td>425 (827 to polyA)</td>
<td>100–240</td>
<td>140</td>
</tr>
</tbody>
</table>
cells transfected with LIGHT a rat anti-LIGHT polyclonal serum for detection. Immunoprecipitates from ligands were resolved on SDS-PAGE and analyzed by Western blot using human HVEM-Fc or TNFR1-Fc and protein G beads. The immobilized sates of the cells were precleared with human IgG and then treated with

An alternate spliced form of LIGHT

RT-PCR analysis of LIGHT expression in the human T cell hybridoma (II-23.D7) revealed a second transcript ~100 nt smaller in size than the full-length LIGHT message that was also inducible with PMA and ionomycin treatment (Fig. 2). DNA sequencing revealed that the smaller transcript contained an internal deletion of 36 amino acids that removes the entire transmembrane domain, referred to as LIGHT ΔTM (accession number AY028261). LIGHT ΔTM transcripts were observed at levels considerably lower than the full-length LIGHT transcript (Fig. 2B). A similar pattern of inducible expression was also observed in human PBLs following activation with PMA and ionomycin (Fig. 2C). The detection of LIGHT ΔTM in activated peripheral blood T lymphocytes is supportive of a possible biological role for this isoform of LIGHT in vivo.

Analysis of the genomic sequence of LIGHT, using a splice donor and acceptor consensus site prediction model, revealed a splice donor consensus sequence at the exact nucleotide corresponding to the deletion in LIGHT ΔTM. Therefore, this alternative transcript is generated by joining the cryptic splice donor in exon 1, at nucleotide position 111, to the splice acceptor that defines the beginning of exon 2, at nucleotide position 218, resulting in the removal of 107 nucleotides including the transmembrane domain in exon 1 (Fig. 3). Thus, direct genetic evidence from splice donor-acceptor prediction algorithms supports alternative

cDNAs have been cloned of 1169 nt (1) and 1491 nt (9). Therefore, it is likely that the full-length transcript has yet to be defined. Analysis of the nucleotide sequences 5’ of the LIGHT gene up to the C3 region of the C3 gene revealed three potential consensus TATAA elements at nucleotide positions −1979, −1762, and −839 relative to the ATG of the first exon. In addition, a combination of consensus motifs for sequence-specific DNA binding proteins, consistent with the inducibility of LIGHT by TCR signaling, are also present. These motifs, among others, include AP-1, NF-κB, and Oct-1 binding sites, which are all present in the highly TCR-inducible IL-2 promoter (33). Usage of the TATAA element at nucleotide position −1762 would yield transcripts of ~2.8 kb; this transcript size would be more consistent with those observed

FIGURE 4. Analysis of LIGHT ΔTM expression in 293T cells. A, LIGHT ΔTM binds HVEM but is not secreted. 293T cells were transiently transfected with cDNA encoding LIGHT ΔTM, soluble FLAG-tagged LIGHT (LIGHTt66) containing the VCAM signal peptide for secretion, or empty pcDNA3.1. Spent culture supernatants and nonionic detergent lysates of several cells were pre-treated with human IgG and then treated with human HVEM-Fc or TNFR1-Fc and protein G beads. The immobilized ligands were resolved on SDS-PAGE and analyzed by Western blot using a rat anti-LIGHT polyclonal serum for detection. Immunoprecipitates from cells transfected with LIGHT ΔTM (lanes 3, 6, and 7), soluble LIGHTt66 (lanes 1, 4, and 9), or empty pcDNA3.1+ (lanes 2, 5, and 8). Purified soluble LIGHTt66 (10 ng) was included (lane 10) as a size standard. Detergent lysates (5 × 10⁶ cell equivalents) from each cell was analyzed to assess relative amount of expressed protein (lanes 7–9). B, LIGHT ΔTM is not glycosylated. Immunoprecipitates from the NP40 cell lysates of 293T cells transfected with soluble LIGHT ΔTM (lanes 1–3), pcDNA3.1+ alone (lanes 4–6), LIGHTt66 (lanes 7–9), or full-length LIGHT (lanes 11–13) encoding plasmids are shown. Detergent lysates were pre-cleared with an isotype control and then immunoprecipitated with mouse anti-human LIGHT Ab and protein G. The immunoprecipitates were digested with either endo H (lanes 2, 5, 8, and 12) or PNaseF (lanes 3, 6, 9, and 13), or were left untreated (lanes 1, 4, 7, and 11); purified soluble LIGHT (lane 10).

FIGURE 5. LIGHT is shed from the surface of 293T cells. 293T cells were transiently transfected with plasmids encoding either membrane LIGHT or empty pcDNA3.1+. HVEM-Fc was used to precipitate LIGHT from the tissue culture supernatant (lane 1) or cell lysate (lane 3) of membrane LIGHT-transfected 293T cells. The precipitates were resolved by SDS-PAGE and Western blot analysis using a rat anti-LIGHT polyclonal serum as probe. TNFR1-Fc was used to control for nonspecific binding to membrane LIGHT (lane 2) or supernatants (lane 4). An equivalent amount (5 × 10⁴ cell equivalents) from each transfected cell lysate was loaded in lanes 5 and 7. Purified recombinant soluble LIGHTt66 (10 ng) (lane 6) used as a molecular mass marker.
splicing of \textit{LIGHT} as the mechanism for the generation of the smaller \textit{LIGHT} transcript detected by RT-PCR.

A cDNA encoding \textit{LIGHT} \textit{\Delta TM} was cloned into the mammalian expression vector (pCDNA3.1\textsuperscript{+}) to study its biologic activity. 239T cells transfected with this construct expressed a 28-kDa protein that reacts with a polyclonal rat anti-human \textit{LIGHT} antiserum by Western blot analysis (Fig. 4A). In addition, HVEM-Fc fusion protein, but not TNFR1-Fc, binds a portion of the expressed \textit{LIGHT} \textit{\Delta TM} suggesting that it assembles as a trimer.

Typical of other TNF-related ligands, \textit{LIGHT} is a type II (cytoplasmic amino terminus) transmembrane glycoprotein. However, in the absence of a transmembrane domain, \textit{LIGHT} \textit{\Delta TM} no longer contains the necessary stop-transfer signal for proper membrane topology and may either be routed to the cytosol or secreted. To determine the cellular location of \textit{LIGHT} \textit{\Delta TM}, nonionic detergent lysates or spent supernatants from \textit{LIGHT} \textit{\Delta TM}-transfected 293T cells were analyzed by precipitation with HVEM-Fc. \textit{LIGHT} \textit{\Delta TM} was not detectable in the culture supernatant (Fig. 4A, lane 3), but was precipitated from the cell extract (Fig. 4A, lane 7) as a single band of \textasciitilde28 kDa. In contrast, an N terminus-truncated soluble form of \textit{LIGHT} (\textit{LIGHT}t66), replaced with the signal peptide from VCAM1 to direct its secretion, was readily detected in the supernatant (Fig. 4A, lane 1) and cell lysate (Fig. 4A, lane 4) as multiple bands, which is suggestive of glycosylation intermediates.

The extracellular domain of \textit{LIGHT} contains a single predicted site for N-linked glycosylation at amino acid position Asn102. Thus, digestion with selected glycohydrolases should provide a diagnostic picture of whether \textit{LIGHT} \textit{\Delta TM} is processed as secreted \textit{LIGHT}t66 or membrane \textit{LIGHT} (Fig. 4B). Enzyme H cleaves high mannose structures and some hybrid oligosaccharides characteristically of glycoproteins that are in progress through the secretory pathway, whereas PNGase F cleaves nearly all types of N-glycans at the asparagine residue of N-linked glycoproteins. \textit{LIGHT} \textit{\Delta TM} displayed no shift in mobility when treated with either endo H or PNGase F (Fig. 4B, lanes 1–3). By contrast, \textit{LIGHT}t66 in the cell-associated immunoprecipitates (lanes 7–9) was shifted in apparent molecular mass by these glycohydrolases. Digestion of full-length \textit{LIGHT} by either endo H or PNGase F shifted the apparent mass indicating that membrane-bound \textit{LIGHT} was glycosylated (Fig. 4, lanes 11–13). Together these results indicate that \textit{LIGHT} \textit{\Delta TM} is likely moving into a cytosolic compartment, whereas \textit{LIGHT}t66 and membrane \textit{LIGHT} are processed normally through the secretory pathway. The identification of \textit{LIGHT} \textit{\Delta TM} is the first example of the production of a soluble TNF family ligand by alternative splicing. Likewise, it is unique that \textit{LIGHT} \textit{\Delta TM} is not destined for secretion, but is probably retained in the cell cytosol and translated on free ribosomes, unavailable for processing by glycosyltransferases in the endoplasmic reticulum, and thus matures in a compartment distinct from transmembrane \textit{LIGHT}. The function of \textit{LIGHT} \textit{\Delta TM} in the cytosol remains to be ascertained.

\textit{LIGHT} is shed

A metalloprotease cleavage site in FasL (34) is also present in \textit{LIGHT} (residues 81–84). Although previous immunoprecipitations failed to detect a shed form of \textit{LIGHT} produced by activated II-23 T cells (1), the possibility could not be dismissed that other cell types might be capable of shedding \textit{LIGHT}. Consistent with the size of the predicted shed protein, 293T cells transfected with full-length transmembrane \textit{LIGHT} displayed a \textasciitilde26-kDa form in the supernatant (Fig. 5). Furthermore, only the 30-kDa full-length form of \textit{LIGHT} was present in the cellular fraction. This pattern is predicted for a molecule cleaved on the external side of the membrane, as occurs for TNF (35). The shed form of \textit{LIGHT} binds HVEM-Fc suggesting that it is a functional ligand. However, with FasL the soluble form generated by shedding is not capable of inducing apoptosis (34). Bearing on this result, recombinant \textit{LIGHT} truncated near the cleavage site is relatively unstable compared with the \textit{LIGHT}t66 form (C. Ware, unpublished observations), suggesting the possibility that shedding of membrane \textit{LIGHT} could be a mechanism of inactivation.

Together, these results demonstrate that \textit{LIGHT} exists in several distinct molecular forms, which are directed to distinct cellular compartments, including the extracellular space, the membrane, and the cytosol. The realization that \textit{LIGHT} is tightly linked to molecules of immunologic importance, including \textit{41BB}, \textit{CD27L}, and \textit{C3}, which reside in an MHC paralog, suggests that this region could play an undisclosed role in genetically linked immune pathology, which may now be possible to define.

\textbf{Acknowledgments}

The assistance provided by the General Clinical Research Center of Scripps Research Institute and Randi Vita for isolation of T cells and G. Valkirs and T. Miyakaya for the anti-LIGHT Omniclone are gratefully appreciated.

\textbf{References}


