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LIGHT, a New Member of the TNF Superfamily, and Lymphotoxin α Are Ligands for Herpesvirus Entry Mediator

Davide N. Mauri,[1,8] Reinhard Ebner,[5,8] Rebecca I. Montgomery,[2,8] Kristine D. Kochel,[1] Timothy C. Cheung,[1] Guo-Liang Yu,[5] Steve Ruben,[5] Marianne Murphy,[6] Roselyn J. Eisenberg,[3] Gary H. Cohen,[4] Patricia G. Spear,[2] and Carl F. Ware[1,7]

[1]Division of Molecular Immunology
La Jolla Institute for Allergy and Immunology
San Diego, California 92121
[2]Department of Microbiology-Immunology
Northwestern University Medical School
Chicago, Illinois 60611
[3]Department of Pathobiology
School of Veterinary Medicine
[4]Department of Microbiology
School of Dental Medicine
University of Pennsylvania
Philadelphia, Pennsylvania 19104-6002
[5]Department of Protein Therapeutics
[6]Department of Cell Biology
Human Genome Sciences
Rockville, Maryland 20850

Summary

Herpes simplex virus (HSV) 1 and 2 infect activated T lymphocytes by attachment of the HSV envelope glycoprotein D (gD) to the cellular herpesvirus entry mediator (HVEM), an orphan member of the tumor necrosis factor receptor superfamily. Here, we demonstrate that HVEM binds two cellular ligands, secreted lymphotoxin α (LTα) and LIGHT, a new member of the TNF superfamily. LIGHT is a 29 kDa type II transmembrane protein produced by activated T cells that also engages the receptor for the LTαβ heterotrimer but does not form complexes with either LTα or LTβ. HSV1 gD inhibits the interaction of HVEM with LIGHT, and LIGHT and gD interfere with HVEM-dependent cell entry by HSV1. This characterizes herpesvirus gD as a membrane-bound viokine and establishes LIGHT-HVEM as integral components of the lymphotoxin cytokine-receptor system.

Introduction

Herpes simplex virus (HSV) 1 and 2 cause recurrent infections that range in severity from benign to serious. HSV emerges from latency in neurons to infect the skin and other tissues in the presence of a competent cellular immune system. The virus initiates infection by binding cell surface glycosaminoglycans and cellular proteins (Spear, 1993). Recently, a cellular HSV entry mediator (HVEM) was identified by expression cloning (Montgomery et al., 1996). HVEM is a type I transmembrane protein with a cysteine-rich extracellular domain that exhibits significant homology with receptors for the tumor necrosis factor (TNF)–related cytokines (Smith et al., 1994; Ware et al., 1995). Antibodies to HVEM inhibit HSV infection of activated T lymphocytes but not of some other cell types (Montgomery et al., 1996). Studies with isolated proteins revealed that HVEM interacts directly with the virus envelope glycoprotein D (gD) (Whitbeck et al., 1997). These results raised the possibility that infection of T cells by HSV could serve as an anti-immune response mechanism. The unknown nature of the cellular ligand(s) for HVEM has precluded a clear understanding of its role in immune physiology, including whether the binding HSV gD protein is relevant to the immunologic function of HVEM and the pathology of herpesvirus infection. We reasoned that a cellular ligand for HVEM should display characteristics similar to the TNF-related ligands, such as LTα or LTβ, because of the sequence homology of HVEM with members of the TNF receptor superfamily.

The TNF-related cytokine-receptor systems have emerged as regulators of the afferent and effector phases of the immune response (Smith et al., 1994; Tracey and Cerami, 1994) and of elements involved during development of lymphoid organs (Matsumoto et al., 1997b). TNF and lymphotoxin α (LTα) are compact trimers assembled from subunits that have an anti-parallel β-sandwich topology, a feature that defines this protein family (Eck and Sprang, 1989; Jones et al., 1989). The binding site for elongated cell surface receptors lies at the interface between adjacent subunits of the trimer (Banner et al., 1993). Ligand binding initiates cellular responses by clustering receptors that, in turn, activate distinct signaling pathways involved in cell death or survival and differentiation. The genes for TNF, LTα, and LTβ are tightly linked within the major histocompatibility complex (MHC) on chromosome 6 (Browning et al., 1993) or chromosome 17 in the mouse (Lawton et al., 1995), although the genes for other ligands in this superfamily are dispersed across the genome. TNF, like most of its relatives, is a type II transmembrane protein (Pennica et al., 1984) that is released from the cell surface by proteolysis (Black et al., 1997). The exception is LTα, which lacks a transmembrane domain and is exclusively secreted as a homotrimer (Gray et al., 1984). However, when expressed as a surface protein, LTα associates with LTβ (Androlewicz et al., 1992; Browning et al., 1993), a type II transmembrane glycoprotein, as heterotrimers of α1β2 and α2β1 subunit ratios (Androlewicz et al., 1992; Browning et al., 1996).

Lymphotoxins and TNF exhibit relatively complex receptor-binding patterns when compared to the monogamous pairing of the other related ligand–receptor systems. LTα and TNF both bind and signal through two receptors, the 55–60 kDa TNF receptor (TNFR60; CD120a or type 1) (Loetscher et al., 1990; Schall et al., 1990) and the 75–80 kDa TNFR (TNFR80; CD120b or type 2) (Smith et al., 1990). By contrast, the surface LTα1β2 complex is recognized specifically by the LTβ receptor (LTβR) (Crowe et al., 1994b), which does not bind either LTα

[7]To whom correspondence should be addressed (e-mail: carl_ware@liai.org).
[8]These authors made equal contributions.

or TNF (Crowe et al., 1994b), although both TNFRs bind the LTα2β1 heterotrimer (Crowe et al., 1994b; Browning et al., 1995), a ligand bound weakly by the LTβR. Although this apparent complexity of receptor specificity suggests functional redundancy, deletions of LTα, LTβ, and TNF genes in mice have revealed specific roles for each ligand in development and function of the immune system. LTα (De Togni et al., 1994; Banks et al., 1995) and LTβ (Alimzhanov et al., 1997; Koni et al., 1997) knockout mice develop without lymph nodes and Peyer's patches and fail to form germinal centers necessary for antibody production to T cell–dependent antigens (Matsumoto et al., 1996b; Fu et al., 1997). TNF and TNFR60 knockout mice have normal lymph nodes but fail to form germinal centers (Pasparakis et al., 1997). Lymphoid organogenesis is largely attributed to signaling by LTα1β2 complex through the LTβR (Ettinger et al., 1996; Rennert et al., 1996), although differences in the phenotypes of the LTβ and LTα knockout mice have suggested the possibility of another receptor(s) involved in these developmental processes (Koni et al., 1997).

Here, we demonstrate that HVEM binds two ligands: LTα and a novel membrane protein whose sequence identifies it as a new member of the TNF superfamily. The new cytokine, termed LIGHT, is homologous to *l*ymphotoxins, exhibits *i*nducible expression, and competes with HSV *g*lycoprotein D for *H*VEM, a receptor expressed by *T* lymphocytes. Identification of the cellular ligands for HVEM provides an essential step forward in understanding the strategies used by herpesvirus to evade the immune system.

Results

Ligand Specificity of HVEM

Activation of T cells results in transient expression of several TNF-related ligands on the cell surface (Ware et al., 1995). To detect a ligand(s) for HVEM, a surrogate receptor was constructed with the extracellular domain of HVEM and the Fc region of human IgG (HVEM:Fc). Activation of the $CD4^+$ II-23 T cell hybridoma with a combination of the calcium ionophore, ionomycin, and phorbol myristate acetate (PMA) but not with PMA alone induced specific staining with HVEM:Fc (Figure 1A). Activated $CD4^+$ and $CD8^+$ subsets of T lymphocytes derived from human peripheral blood (Figure 1B) stained with HVEM:Fc, indicating that the putative ligand is expressed by transformed and normal cells. PMA alone induces the expression of LTα, LTβ, and TNF on the II-23 cell line (Ware et al., 1992). In contrast to HVEM:Fc, staining of II-23 cells with LTβR:Fc increased with PMA treatment but was substantially reduced (~60%) after treatment with a combination of PMA and ionomycin (Figure 1A). After activation with PMA, II-23 cells concurrently express membrane TNF and a small amount of LTα2β1 complex as detected by the TNFR60:Fc (Crowe et al., 1994b; Browning et al., 1995). The level of membrane TNF also increased with the addition of ionomycin (data not shown).

To determine whether HVEM might bind to TNF or LTαβ complexes, LTβR:Fc and TNFR:Fc (Fc of human IgG1) were used as competitive inhibitors of HVEM:Fc constructed with rabbit IgG (Montgomery et al., 1996).

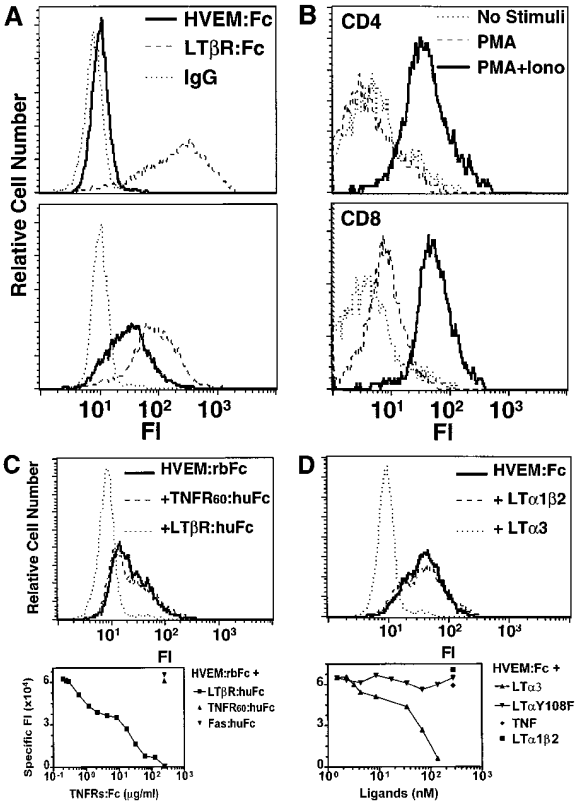

Figure 1. HVEM Recognizes a Membrane-Bound Ligand on Activated T Cells

(A) Expression of an HVEM ligand by activated II-23 T hybridoma cells. (Top) II-23 cells were activated for 4 hr at 37°C with phorbol ester (100 ng/ml) or (bottom) PMA (100 ng/ml) and ionomycin (1 μg/ml). Cells were incubated for 30 min at 4°C with HVEM:Fc, mLTβR:Fc, or human IgG at 5 μg/ml and then stained with goat anti-huIgG-PE. Each histogram represents 10^4 events.

(B) HVEM:Fc binds activated T cells from human peripheral blood. Peripheral blood mononuclear cells were activated with anti-CD3 for 5 days in medium with IL-2. Cells were restimulated with PMA or PMA and ionomycin (Iono) for 4 hr and then dual stained with FITC-CD4 or FITC-CD8, and HVEM:Fc detected with goat anti-huIgG-PE as described above.

(C) HVEM:Fc binding is competed by LTβR:Fc. (Top) activated II-23 cells (PMA and ionomycin as described in [A]) were preincubated with mLTβR:Fc or TNFR60:Fc (100 μg/ml) for 30 min at 4°C. HVEM:Fc (rabbit) (2 μg/ml) was then added, incubated for 30 min, and the cells stained with goat anti-rabbit IgG-PE. Rabbit IgG was used to determine background staining. (Bottom) dose-dependent inhibition of HVEM:Fc binding by LTβR:Fc. Binding of HVEM:Fc to activated II-23 cells was competed with graded concentrations of mLTβR:Fc, TNFR60, Fas:Fc, or IgG as described above.

(D) HVEM:Fc binding is competed by LTα homotrimer. (Top) II-23 cells were activated, and HVEM:Fc was preincubated with recombinant LTα, LTαY108F, or LTα1β2 (100 nM) for 30 min at 4°C. The mixture was added to activated II-23 cells and then stained with anti-huIgG-PE. Fluorescence staining with HVEM:Fc+LTα was equal to background with normal IgG (data not shown). (Bottom) dose dependence of LTα competition for HVEM:Fc binding to activated II-23 cells as determined by flow cytofluorometry.

The LTβR:Fc and HVEM:Fc but not TNFR60:Fc competed for the binding of HVEM:Fc (rabbit) (Figure 1C). The related receptors, Fas:Fc and TNFR80:Fc, did not compete with HVEM:Fc (data not shown). Surprisingly, the LTα homotrimer (which binds TNFR) but not TNF or LTα1β2 (the latter binds LTβR) strongly competed for

HVEM:Fc binding (Figure 1D). However, a mutant of LTα at tyrosine 108 to phenylalanine (Y108F) that is unable to engage TNFR (Goh et al., 1991; Williams-Abbott et al., 1997) failed to compete for HVEM:Fc binding (Figure 1D). The binding curve for LTβR:Fc suggests the presence of high (~10 nM) and low affinity (~50–100 nM) components, whereas the LTα competition curve showed 50% inhibition at approximately 50–80 nM. These results indicate that the putative HVEM ligand(s) have characteristics in common with the LTαβ heterotrimers and LTα, but these features clearly distinguish it from LTα1β2 and TNF. Expression of LTαβ heterotrimers in insect cells using recombinant baculovirus (Williams-Abbott et al., 1997) failed to reconstitute the membrane HVEM ligand but did form ligands for LTβR:Fc and TNFR:Fc (data not shown). Together, these results suggest that the membrane protein recognized by HVEM:Fc is similar to both lymphotoxins but distinct from the known forms of LTαβ. A biochemical approach was used to investigate the structure of the HVEM:Fc-binding protein.

II-23 cells secrete LTα after activation with PMA (Ware et al., 1992; Crowe et al., 1994b). HVEM:Fc and TNFR60:Fc precipitated proteins of 23–25 kDa from supernatants of [^{35}S]methionine- and [^{35}S]cysteine-labeled II-23 cells stimulated with PMA and ionomycin (Figure 2A, lanes 2–5). Secreted LTα displays a similar pattern of bands that is heterogeneous due to glycosylation (Browning et al., 1991). TNFR60:Fc but not HVEM:Fc also precipitated a trace amount of a 17 kDa protein identical in size to secreted TNF. LTβR:Fc, as expected, did not bind any secreted proteins; however, proteins of 23–25 kDa (LTα) and 33 kDa (LTβ) characteristic of the LTα1β2 complex were precipitated from the cell-associated fraction (lane 6) of PMA-activated cells. By contrast, when the stimulus included ionomycin and PMA, LTβR:Fc precipitated a major band at 30 kDa with nearly complete absence of LTα and LTβ (lane 7). TNFR60:Fc precipitated a 23 kDa protein identical in size to the LTα precursor (lane 8), whereas HVEM:Fc precipitated both 30 kDa and 23 kDa proteins (lane 9). Three different receptor blocking–monoclonal antibodies to LTβ failed to remove the 30 kDa protein from the extract prior to the addition of HVEM:Fc, indicating this protein is antigenically unrelated to LTβ (Figure 2B, lanes 3, 4, and 6). However, anti-LTα antibodies removed the 23 kDa band from the extracts, indicating relatedness to LTα (lanes 2 and 5). The inability of LTα antibodies to simultaneously preclear both the 30 kDa and 23 kDa bands demonstrates that these proteins are not associated with each other, unlike the LTαβ heterotrimers that readily coimmunoprecipitate with antibody to either subunit (Androlewicz et al., 1992; Browning et al., 1995). Peptide mapping also showed a distinct pattern for the cell-associated HVEM-binding protein when compared to LTβ, indicating this protein is not likely to be an isoform of LTα or LTβ (data not shown). These results indicate that the membrane-associated HVEM ligand is antigenically distinct from LTα and LTβ and displays receptor-binding properties that distinguish it from other known TNF-related ligands.

The identity of the secreted 23–25 kDa HVEM ligand as LTα was confirmed by the specific precipitation of recombinant LTα but not soluble TNF or LTα1β2 by

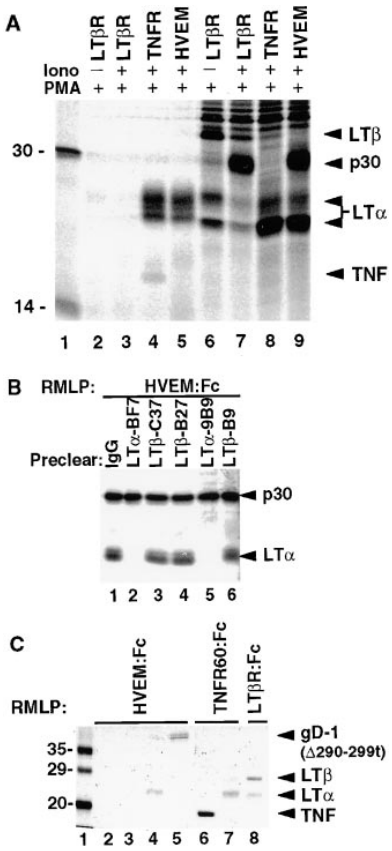

Figure 2. Biochemical Identification of HVEM Ligands

(A) Receptor-mediated ligand precipitation with HVEM:Fc. II-23 cells activated for 4 hr with PMA or PMA and ionomycin (as in Figure 1) were labeled with [^{35}S]methionine and [^{35}S]cysteine. The supernatant or detergent extract was precleared with human IgG (10 μg) and protein G-Sepharose beads and then the indicated receptor:Fc fusion proteins (10 μg/ml) were then added to the samples and precipitated with protein G beads. Labeled proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and phosphoimage (pixel range 6–200). Lane 1, molecular weight markers; lanes 2–5, culture supernatants; lanes 6–9, detergent extracts.

(B) Antigenic identity of HVEM ligands. Cellular extracts prepared as in (A) were first precleared with 10 μg of mouse IgG (lane 1) or the indicated monoclonal antibodies to LTα (lanes 2 and 5) or LTβ (lanes 3, 4, and 6), and then HVEM:Fc was added to precipitate ligands. The proteins bound to HVEM:Fc were then resolved by SDS-PAGE and detected by phosphoimage.

(C) HVEM binds LTα and gD-1. HVEM:Fc, TNFR60:Fc, or LTβR:Fc (10 μg) in 1 ml of 1% BSA, 0.1% NP-40, 0.15 M NaCl, Tris, (pH 7.4) buffer was mixed with 1 μg of recombinant soluble TNF (lanes 2 and 6), LTα1β2 (lanes 3 and 8) or gD-1(Δ290-299t) (lane 5), or LTα (lanes 4 and 7), incubated for 1 hr at 4°C, and then precipitated with protein G beads. The bound proteins were resolved by SDS-PAGE (12%) and stained with Coomassie blue.

HVEM:Fc (Figure 2C). This distinguishes the ligand specificity of HVEM from both TNFRs and indicates HVEM binds at least two ligands, LTα and a distinct membrane protein.

LIGHT, a Membrane-Anchored HVEM Ligand

To identify the membrane ligand for HVEM, several cDNAs encoding proteins with sequence homology to the TNF superfamily, identified by sequencing of an activated T cell library, were transfected into HEK293 cells

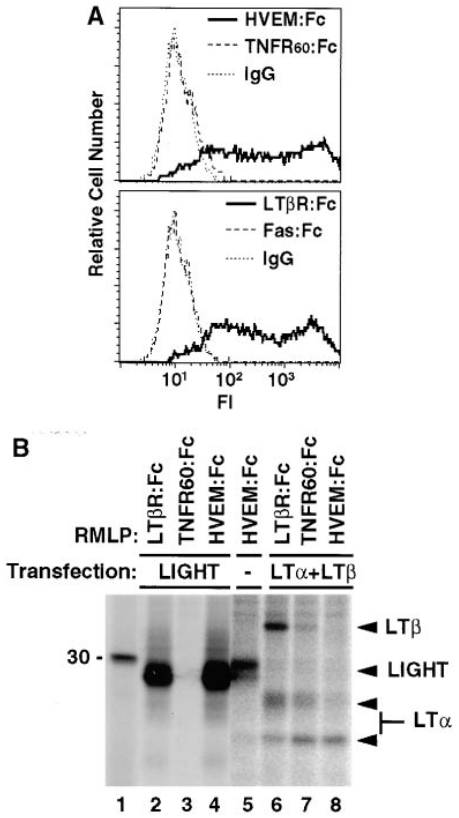

Figure 3. Identification of the HVEM Surface Ligand

(A) HEK293 cells (10^6) were transfected with 5 μg LIGHT cDNA (pCDNA3 with CMV promoter) using the calcium phosphate method. After 24 hr transfection, cells were harvested with 20 mM EDTA in PBS and incubated with HVEM:Fc, mLTβR:Fc, TNFR:Fc, Fas:Fc, or IgG and stained with anti-huIgG-PE. Mock-transfected cells did not stain with any Fc fusion protein.

(B) HEK293 cells transfected with LIGHT cDNA or cotransfected with LTα and LTβ cDNAs (pCMD8 with CMV promoter) (as described above) were metabolically labeled and the extracts precipitated with mLTβR:Fc (lanes 2 and 6), TNFR60:Fc (lanes 3 and 7), HVEM:Fc (lanes 4, 5, and 8) as described above. For comparison, in lane 5, the HVEM:Fc precipitation was from metabolically labeled activated II-23 cells.

and assayed for HVEM:Fc binding by flow cytometry. Transfection of one of these cDNAs resulted in specific staining of HEK293 cells by HVEM:Fc and LTβR:Fc but not TNFR60:Fc or Fas:Fc (Figure 3A). A 28–29 kDa protein (observed as a doublet) was specifically precipitated by HVEM:Fc and LTβR:Fc but not by TNFR60:Fc from extracts of metabolically labeled HEK293 cells transfected with this cDNA (Figure 3B). The primary structure of this protein predicted from the cDNA sequence (Figure 4A) contains 240 amino acids with an N-terminal cytosolic domain of 37 residues that precedes a stretch of 22 hydrophobic residues characteristic of a type II transmembrane protein. LIGHT exhibits significant sequence homology with the C-terminal receptor-binding domains of LTβ (34% identity), Fas Ligand (31%), 41BBL (29%), TRAIL (28%), LTα (27%), TNF (27%), and CD40L (26%) (Figure 4B). The ligands for CD30, CD27, and OX-40 exhibit the weakest homology (12%–18% identity, not shown in this alignment). Sequence homology is primarily limited to the residues forming β-strand scaffold, suggesting this protein, like LTα, CD40L, and TNF, folds into an anti-parallel β-sandwich structure and assembles as a trimer. Similarity of LIGHT with the lymphotoxins outside the scaffold regions is seen in the conservation of tyrosine 173 located in the D-E loop, a contact region in LTα for TNFR60, and as shown (Figure 1D) when mutated to phenylalanine ablates the ability of LTα to compete with HVEM. The single N-linked glycosylation site (N102) predicted for this protein lies within the other major receptor-binding loop (A-A′′). These results strongly indicate this cDNA encodes a similar, if not identical, protein to the HVEM ligand expressed by activated T cells.

By Northern blot analysis, a 2.5 kb transcript for LIGHT is expressed predominantly in spleen but also in brain where a secondary transcript of 3.5 kb is seen (Figure 5). A weak hybridization signal was detected in peripheral lymphoid tissues and in heart, placenta, liver, lung, appendix, and kidney. The mRNA encoding LIGHT was not detected in fetal tissues and organs or in many endocrine glands and tumor lines of nonhemopoietic and myeloid origin (data not shown).

HSV gD Is a Virokine

The possibility that HSV gD might function as an antagonist of the HVEM cellular ligands was suggested by the binding of HSV gD-1 protein to HVEM (Whitbeck et al., 1997; also see Figure 2C). This was formally tested using soluble forms of gD as antagonists of HVEM binding to the surface of activated II-23 cells or HEK293 cells transfected with LIGHT cDNA. Soluble gD-1(306t) and gD-1(Δ290-299t), a mutant with enhanced binding for HVEM (Whitbeck et al., 1997) and the ability to block HSV infectivity (Nicola et al., 1996), were both effective at inhibiting HVEM:Fc binding to the surface of activated II-23 cells (Figure 6A) and LIGHT-transfected cells (Figure 6B). The effective inhibitory concentration of gD-1 proteins correlated with their affinity for HVEM (Whitbeck et al., 1997). The competition curve with gD-1 (Δ290-299t) on II-23 cells suggests the presence of high- and low-affinity components. The biphasic curve is also apparent with LIGHT-transfected cells, although the high affinity site is a minor component. This result suggests that cooperative binding interactions occur between HVEM:Fc and the surface ligand, rather than multiple ligands with different affinities for HVEM:Fc. The binding of LTβR:Fc or TNFR60:Fc to PMA or PMA/ionomycin-activated II-23 cells was not inhibited by gD-1(Δ290-299t), indicating that the binding between HVEM and gD-1 is highly specific (data not shown).

Human cells that express gD-1 can be resistant to HSV1 entry (Campadelli-Fiume et al., 1988; Johnson and Spear, 1989), possibly due to receptor blockade or sequestration of the receptor inside the cell. This interference phenomenon and the results described above suggested that LIGHT or gD-1, if coexpressed with HVEM, might interfere with HSV1 entry of cells via HVEM. To test this, resistant CHO cells were cotransfected with an HVEM-expressing plasmid, which confers susceptibility to HSV1 entry, and a plasmid expressing LIGHT or gD-1,

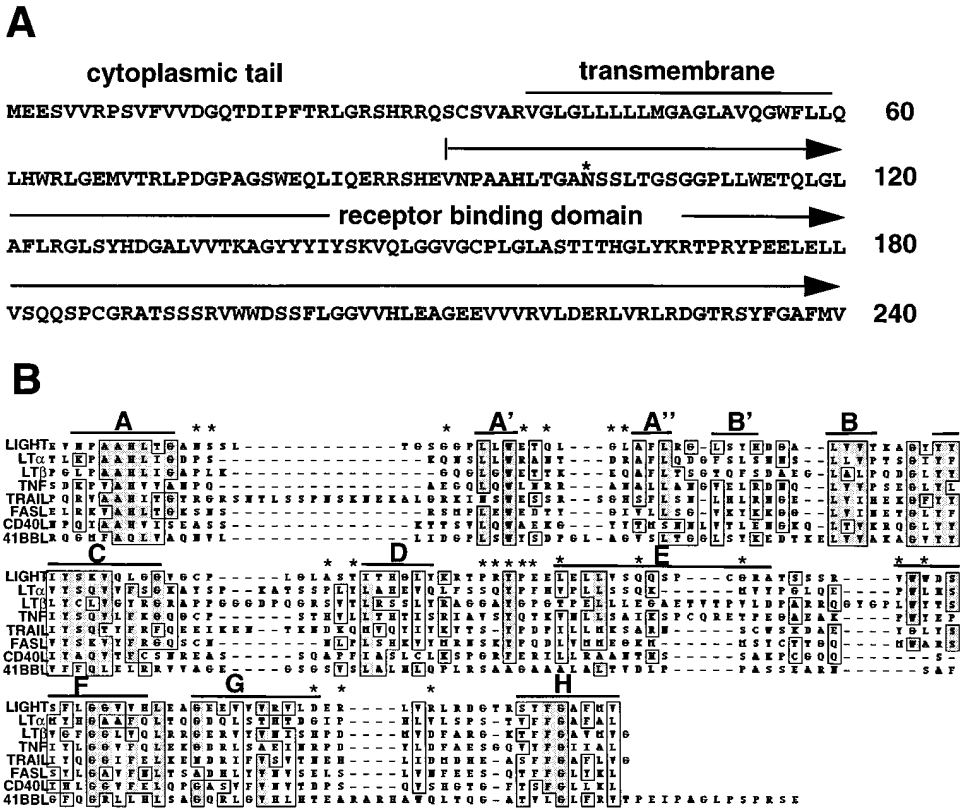

Figure 4. Sequence of LIGHT and Alignment with TNF Superfamily

(A) Amino-acid sequence deduced from the cDNA sequence. The asterisk (*) indicates the position of the predicted N-glycosylation site. (B) Alignment of LIGHT with TNF-related ligands. Sequences were aligned with ClustalW (Pam250 matrix) (Macvector). This alignment excludes ligands for CD27, CD30, and Ox-40. Asterisks, the contact residues in LTα for TNFR60; bars, the β-strands that form the scaffold in LTα as designated by convention (Eck et al., 1992). Homology regions are boxed with identical residues shaded.

or a control plasmid. The cells were then inoculated with various concentrations of recombinant HSV1, KOS-gL86, which expresses β-galactosidase upon viral entry, and enzyme levels were quantitated after 6 hr. (Figure 7). Cotransfection of HVEM with LIGHT or gD-1 but not with control plasmid dramatically inhibited virus entry, whereas LIGHT and control plasmid did not confer susceptibility. These results indicate that LIGHT as well as gD-1 can interfere with HSV1 entry via HVEM.

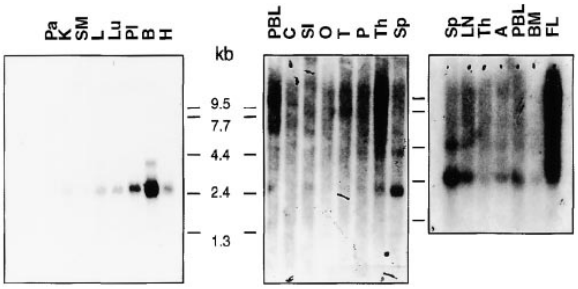

Figure 5. Expression of LIGHT mRNA

Northern blot analysis of poly(A)$^+$ RNA (2 μg/lane) from various human tissues. Numbers on the left indicate the lengths of RNA molecular size standards. RNA from spleen was used as common positive tissue for estimating variation in the hybridization signal. Tissues tested include the following: pancreas (Pa), kidney (K), skeletal muscle (SM), liver (L), lung (Lu), placenta (Pl), brain (B), heart (H), peripheral blood leukocytes (PBL), mucosal lining of colon (C), small intestine (SI), ovary (O), testis (T), prostate (P), thymus (Th), spleen (Sp), lymph node (LN), appendix (A), bone marrow (BM), and fetal liver (FL).

Discussion

The work presented here establishes HVEM and LIGHT as integral components of the LT/TNF cytokine-receptor system. The ligand and receptor interactions in this system show a significant divergence in specificity, although substantial overlap is evident in cognate pairing (eg., LTα binds TNFR60, TNFR80, and HVEM). At present, the majority of evidence indicates that each ligand–receptor pair identified so far functions autonomously in ligand binding and signaling activities. However, the cross-specificity of several receptors certainly suggests that cooperative interactions could occur, and recent evidence with TNFR supports this concept (Pinckard et al., 1997). Our results also demonstrate LIGHT is a previously unrecognized membrane-anchored ligand for LTβR, a finding that establishes a direct relation between LTβR and HVEM. It is not surprising that LTβ and LIGHT are closely related in their primary sequence, suggesting a close functional link exists between these

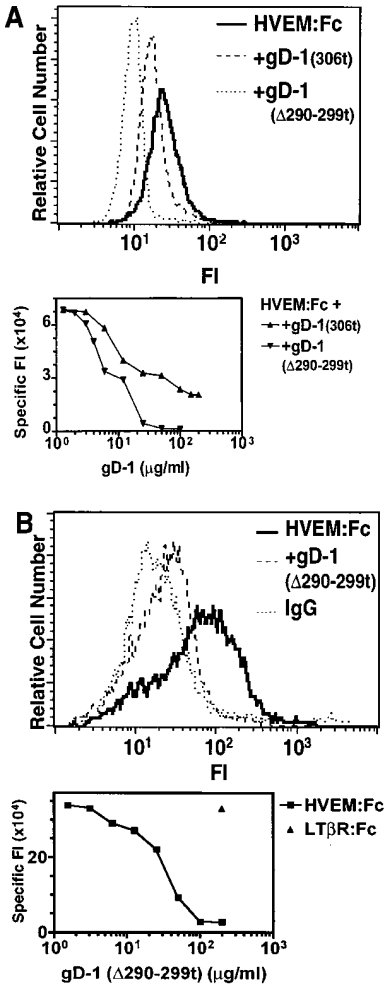

Figure 6. HSV1 gD Inhibits HVEM:Fc Binding to Activated II-23 Cells and LIGHT-Transfected HEK293 Cells

(A) (Top) HVEM:Fc (2 μg/ml) was preincubated for 30 min at 4°C with gD-1(306t) (250 μg/ml) or gD-1(Δ290-299t) (100 μg/ml) and then added to PMA and ionomycin–activated II-23 cells (as in Figure 1A). Background staining was determined with human IgG (data not shown) and is equal to HVEM:Fc+gD-1(Δ290-299t). (Bottom) gD-1 competition binding analysis. Binding of HVEM:Fc to activated II-23 cells was competed with graded concentrations of gD-1(306t) or gD-1(Δ290–299t) as indicated in Figure 1.

(B) (Top) HEK293 cells transfected with LIGHT cDNA were used as in (A).

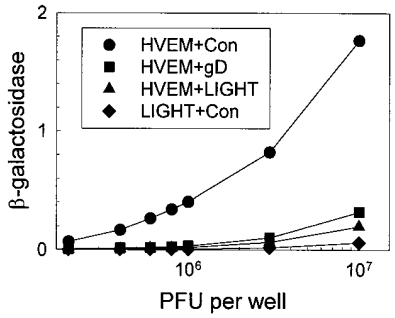

Figure 7. LIGHT Interferes with HVEM-Dependent HSV1 Infection

CHO-K1 cells were cotransfected with the HVEM-expressing plasmid pBEC10 (Montgomery et al., 1996) and control vector pcDNA3, gD-expressing plasmid pRE4 (Cohen et al., 1988), or LIGHT-expressing plasmid pcDNA3-LIGHT; or with the LIGHT-expressing plasmid pcDNA3-LIGHT and control vector pcDNA3. Transiently transfected cells were harvested at 24 hr and plated into 96-well dishes. After overnight incubation, cells were challenged with KOS-gL86 at the indicated doses. After 6 hr of infection, the activity of β-galactosidase expressed from the input viral genome was quantitated. Each point represents the mean of triplicate determinations and individual values were within 10% of the mean. Values reported were obtained 1.5 hr after o-nitrophenyl β-D-galactopyranoside addition.

two systems. However, these ligands differ in several important aspects. For instance, LTβ does not form a homotrimer; instead, it assembles with LTα into a biologically active heterotrimer (Williams-Abbott et al., 1997). In contrast, LIGHT is predicted to be a homoligomer. Also, preliminary evidence indicates the LIGHT gene is not linked to the TNF/LT locus in the MHC (Zhai et al., unpublished data). Furthermore, LTαβ and LIGHT expression on the surface of activated T cells requires different activating signals. PMA is sufficient to induce LTα1β2, whereas Ca ionophore with PMA is required for LIGHT and suppresses the expression of LTα1β2. LIGHT and LTβ mRNAs are expressed in lymphoid tissues; however, LIGHT mRNA is also detected in the brain. Together, the structural and functional differences between LIGHT and LTαβ heteromers strongly suggest that their roles in immune physiology are distinct.

Likewise, HVEM and LTβR share significant properties but have distinguishing features that indicate their physiologic roles are distinct. HVEM and LTβR share the potential to activate common signaling pathways via association with TNFR-associated factors (TRAFs), a family of zinc RING-finger proteins involved in signaling by several members of the TNFR superfamily. HVEM binds TRAF2 and 5 (Marsters et al., 1997; Hsu et al., 1997), as does LTβR (Nakano et al., 1996), and activates NFκB, a transcription factor involved in regulation of proinflammatory and anti-apoptotic genes. LTβR induces apoptosis of an adenocarcinoma line by activating a TRAF3-dependent pathway (VanArsdale et al., 1997). In contrast, we have not identified any cell lines for which HVEM antibodies trigger apoptosis, a finding that correlates with the weak interactions between HVEM and TRAF3 (Marsters et al., 1997; K. D. K. and C. F. W., unpublished data). The interaction between LIGHT and LTβR suggests that LIGHT, in addition to LTα1β2, may activate apoptosis via the LTβR, a possibility currently being investigated.

Collectively, these results indicate the physiologic function(s) of the LIGHT/LTα-HVEM pathway are probably distinct from TNF/LTα-TNFR and LTα1β2/LIGHT-LTβR pathways. Genetic deletions of LTα or LTβ genes in mice have revealed roles for these two genes in the development of lymph nodes and Peyer's patches (De Togni et al., 1994; Banks et al., 1995). Along with TNF and TNFR60, LTα and LTβ also control the formation of germinal centers necessary for immunoglobulin-isotype switching during immune responses in adults (Mariathasan et al., 1995; Matsumoto et al., 1996a, 1996b). Most studies have pointed toward the LTα1β2 and LTβR as the critical cytokine-receptor system controlling these

functions (Crowe et al., 1994b; Ettinger et al., 1996; Rennert et al., 1996; Koni et al., 1997). However, the phenotypes of LTα and LTβ knockout mice differ, particularly the presence of cervical and mesenteric lymph nodes in $LT\beta^{-/-}$ mice (Alimzhanov et al., 1997; Koni et al., 1997). In addition, organization of T and B cell compartments in the spleen is not as severely disrupted in the $LT\beta^{-/-}$ mice as in the $LT\alpha^{-/-}$ mice, a finding suggesting that another receptor may engage the LTα. Moreover, LTα transgenic mice (presumably that overexpress secreted LTα and not LTαβ complexes) grow extralymphatic lymph node–like structures (Picarella et al., 1992; Kratz et al., 1996). The cross-specificity of LIGHT and LTβR and LTα and HVEM makes the LIGHT/LTα-HVEM pathway a likely candidate to account for the differences observed among the lymphotoxin transgenic and knockout mice.

The unique phenotypes of the LTα and LTβ knockouts suggest that LIGHT and $LT\alpha1\beta2$ are not redundant ligands for the LTβR. Several possibilities could account for the distinct activities of these ligands signaling through the same receptor. LIGHT mRNA was not detected in the embryonic tissues, raising the possibility that it may not function during lymph-node genesis. Another possibility is that these two ligands activate different signaling pathways by the LTβR. A potential precedent for this is seen in the $LT\alpha2\beta1$ heteromer that binds both TNFR60 and LTβR but does not activate these receptors in a way that mimics TNF/LTα or $LT\alpha1\beta2$. ($LT\alpha2\beta1$ is a minor form and a functional role, if any, remains to be discovered.) An alternate explanation is that HVEM and LTβR signal in a tissue-specific context. The latter point is intriguing because HVEM and LTβR have distinct tissue expression patterns: HVEM is prominent on T and B lymphocytes (D. N. M., unpublished data; Kwon et al., 1997), whereas LTβR is absent on lymphocytes but is prominent on the stroma in the thymus and spleen and is expressed on a follicular dendritic cell line (Force et al., 1996; Murphy et al., 1998). The ability of LTβR to stimulate expression of adhesion proteins ICAM and VCAM (Hochman et al., 1996) and chemokines (Degli-Esposti et al., 1997) suggests this receptor is important for creating tissue microenvironments suitable for productive T and B cell interactions. Likewise, the expression of HVEM on T and B cells suggests that it may also participate as a regulator of cellular differentiation during formation of germinal centers in peripheral lymphoid organs. Recent studies with Hox-11 knockout mice that are asplenic, as well as alymphoplasia (aly) mice that lack lymph nodes, have revealed an essential role for peripheral lymph organs in anti-virus defenses (Karrer et al., 1997). Although these genes are unrelated to known members of the TNF superfamily, they provide clues that the interaction between herpesvirus and the LT system is not fortuitous.

Our results provide a molecular link connecting the envelope protein gD of HSV1 (an α herpesvirus) with the LT cytokine-receptor system. The ability of gD-1 to specifically compete with LIGHT for binding HVEM but not LTβR suggests that gD-1 may have evolved specifically for the LIGHT–HVEM interaction. The ability of LIGHT to block HVEM-dependent HSV1 infection indicates that gD-1 is a membrane-anchored virokine that mimics LIGHT, and it implicates gD-1 as a potential modifier of HVEM-signaling activities during entry or egress of HSV. Furthermore, expression of gD on the surface of infected cells occurs well before the release of mature virions (Spear, 1993), and such cells could also interact with HVEM-bearing T or B cells. It remains to be seen whether this interaction causes immune modulation of T or B cells either by free HSV virions or by infected cells bearing gD on the cell surface. Of interest is the observation that Epstein-Barr virus (γ herpesvirus) modifies signal transduction by TNFR through the action of the transforming protein LMP1. LMP1 binds members of the TRAF family (Mosialos et al., 1995), activates NFκB, and thus functions like a constitutively activated receptor (VanArsdale et al., 1997). Members of the TNF and TNFR superfamilies are targeted by a number of specific mechanisms (Gooding, 1992) found in a diverse group of viruses that include poxvirus (Schreiber et al., 1997), adenovirus (Shisler et al., 1997), hepatitis C virus (Matsumoto et al., 1997a), and γ herpesvirus (Bertin et al., 1997). These examples provide compelling evidence that counteracting immune defense strategies is essential for virus survival and persistence. It is enticing to speculate that the selective pressure brought to bear upon the immune system by herpesvirus has driven the diversification of the LT ligands and their receptors, and perhaps the collective onslaught of viruses has contributed to the expansion of these cytokines into its present superfamily size. The identification of HVEM as a receptor that interacts with LTα and LIGHT should assist in understanding the complex virus–host relationship.

Experimental Procedures

Cells

The II-23 cell line (D7 subclone) is a human $CD4^+$ T cell hybridoma (Ware et al., 1986) and is maintained in RPMI-1640 with 10% fetal bovine serum (FBS) and antibiotics. II-23 cells were activated with PMA (100 ng/ml) or PMA and ionomycin (1 μg/ml) for 2.5 hr, washed twice with PBS, once with cysteine-methionine–deficient RPMI, and then resuspended in this medium containing 10% dialyzed FBS, 250 μCi [^{35}S]methionine and cysteine, and activating agents for 1.5 hr. The supernatants were harvested, and the cells were lysed in buffer containing 2% NP40, HEPES (pH 7.0), 20 mM EDTA, 150 mM NaCl with leupeptin and aprotinin (at 10 μg/ml), phenylmethylsulfonyl fluoride (1 mM), and iodoacetamide (20 mM). Peripheral blood lymphocytes were obtained from normal donors by Ficoll-Hypaque and cultured in medium supplemented with IL-2 (10 ng/ml) after stimulation with anti-CD3 (OKT3) or phytohemagglutinin.

Receptor:Fc Fusion Proteins, Antibodies, and HSV gD-1

Construction, expression, and purification of the bivalent chimeric proteins formed with the Fc region of human IgG1 and the ligand-binding domains of Fas:Fc (Brunner et al., 1995), TNFR60 (Crowe et al., 1994a), and human LTβR:Fc (Crowe et al., 1994b) have been previously described. The extracellular region of HVEM was generated by polymerase chain reaction using Taq DNA polymerase–amplified sequences from pBEC10 DNA encoding 1-K184 using the forward primer 5′-CGGAGATCTGAGTTCATCCTGCTAGCTGG and reverse primer 5′-ATAGGATCCCTTGGTCTGGTGCTGACATTCC. The amplified HVEM product was ligated in-frame into the baculovirus vector pVL1392 (Pharmingen) containing the human Fc IgG1. A similar construct of HVEM:Fc with Fc region from rabbit IgG1 was produced in CHO cells, purified, and used as an immunogen to produce rabbit anti-HVEM (Montgomery et al., 1996). LTβR:Fc was constructed from mouse LTβR (mLTβR) DNA fragment (Force et al., 1996) by polymerase chain reaction using Taq DNA polymerase with forward primer 5′-GACGTCAGATCTTCCCACCTTTCCTCCTA-3′

and reverse primer 5′-GAACAGAGATCTCATTGCTCCTGGCTCTG-3′ and encodes amino acid residues 1-Met221 of the extracellular domain. LTα and LTαY108F were produced in insect cells using recombinant baculovirus as described (Crowe et al., 1994a; Williams-Abbott et al., 1997). Recombinant soluble LTα1β2 (Browning et al., 1996), TNF (Browning and Ribolini, 1989), and monoclonal antibodies to LTα, BF7, and LTβ, C37, B9, and B27 (Browning et al., 1995) were generous gifts from Jeffrey Browning (Biogen). The immunoprecipitating anti-LTα antibody (clone 9B9) was from Boehringer Mannheim. Purified recombinant wild-type HSV gD-1 protein truncated at position 306 gD-1(306t) and a truncated gD-1 mutant containing an internal deletion between position 290–299 (gD-1Δ290-299t) were produced in baculovirus as previously described in detail (Nicola et al., 1996).

Immunoassays

After stimulation, cells for flow cytometry were harvested, washed, and incubated on ice in Hanks' balanced salt solution with 10% bovine calf serum, 0.1% sodium azide–containing antibodies or TNFR:Fc chimeras at 10 μg/ml. Phycoerythrin-conjugated affinity-purified goat anti-human or rabbit IgG were used to stain Fc fusion proteins. Controls for nonspecific binding included normal human or rabbit IgG. IgG (heat-aggregated, 10 μg/ml) from the appropriate species was included in the buffer to block nonspecific binding. Fluorescence was detected by flow cytometry (FACSCaliber, Becton Dickinson). Fluorescein isothiocynate (FITC)–anti-CD4 and CD8 were obtained from Becton Dickinson. Receptor:Fc binding was determined by calculating the fluorescence intensity as follows: (mean fluorescent channel)(% positive fluorescent events), where a positive event has a fluorescence value of greater than 98% of the value for normal IgG. Specific fluorescence intensity represents the fluorescence intensity after subtraction of the value for control IgG. Antibody and receptor-mediated ligand precipitations were performed as previously described (Crowe et al., 1994b) using receptor:Fc fusion proteins at 10 μg or 5 μg of antibody and isolation of immune complexes with protein G-Sepharose beads.

Isolation of LIGHT cDNA and Northern Blots

Peripheral blood mononuclear cells isolated by Ficoll-Hypaque density centrifugation and depleted of adherent cells, cultured overnight, and then activated with PMA (1 μg/ml) and phytohemagglutinin (2 μg/ml) for 12 hr before RNA isolation (Quiagen oligo dT) and cDNA library construction (λ Uni-AZAP XR; Stratagene). A partial clone was identified by limited homology of its predicted translation product to the extracellular domain of FasL. Several rounds of library screening under high stringency conditions resulted in the isolation of a cDNA clone with a complete reading frame encoding a type II transmembrane protein.

Northern blot analysis of RNA samples was performed using multiple tissue blots (Clontech, Palo Alto, CA; Clontech 7770-1, 7750-1, 7751-1, 7754-1, 7755-1, 7756-1, 7757-1, 7759-1, 7760-1, 7766-1, and 7768-1). Hybridization was performed overnight in Hybrisol solution (Oncor, Gaithersburg, MD), preheated to 42°C before use, followed by two subsequent washes in 2× SSC/0.1% SDS and 0.2× SSC/0.1%SDS at the same temperature. Double-stranded cDNA probes, used at a minimum specific activity of 5×10^8 cpm/mg, were generated by restriction digestion, ^{32}P-labeled using the Rediprime random primer labeling system (Amersham, Arlington Heights, IL), and purified with NucTrap ion exchange pushcolumns (Stratagene, La Jolla, CA).

Infectivity Assays

Infectivity assays were based on quantitation of β-galactosidase expressed from the HSV1(KOS)gL86 genome (KOS-gL86) (Montgomery et al., 1996). Briefly, CHO-K1cells (American Type Culture Collection; Rockville, MD) were cotransfected with a 1:1 mass ratio of the indicated plasmids using LipofectAMINE (GIBCO; 1.5 μg of total plasmid and 5 μl of LipofectAMINE per 35 mm culture) in Opti-MEM medium (GIBCO). At 24–36 hr posttransfection, cells were harvested with PBS containing 2 mM EDTA, resuspended in Ham's F12 medium supplemented with 10% FBS and antibiotics, and inoculated into 96-well dishes ($2–4 \times 10^4$ cells/well). Cells were incubated overnight and then challenged with the β-galactosidase reporter virus KOS-gL86. Infections and quantitation of infection using the β-galactosidase substrate o-nitrophenyl β-D-galactopyranoside (3 mg/ml) were performed as described previously (Montgomery et al., 1996).

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