Discrete Signaling Regions in the Lymphotoxin-β Receptor for Tumor Necrosis Factor Receptor-associated Factor Binding, Subcellular Localization, and Activation of Cell Death and NF-κB Pathways*

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Lymphotoxin-β (LTβ) receptor (LTβR), a member of the tumor necrosis factor receptor superfamily, is essential for the development and organization of secondary lymphoid tissue. Wild type and mutant LTβR containing successive truncations of the cytoplasmic domain were investigated by retrovirus-mediated gene transfer into HT29.14s and in 293T cells by transfection. Wild type receptors accumulated in perinuclear compartments and enhanced responsiveness to ligand-induced cell death and ligand-independent activation of NFκB p50 dimers. Comunnonprecipitation and confocal microscopy mapped the TRAF3 binding site to amino acids PEEGDPG at position 389. However, LTβR truncated at position Pro379 acted as a dominant positive mutant that down-modulated surface expression and recruited TRAF3 to endogenous LTβR. This mutant exhibited ligand-independent cell death and activated NFκB p50 dimers. By contrast, truncation at Gly335 created a dominant-negative mutant that inhibited ligand-induced cell death and activation of NFκB p50/p65 heterodimers. This mutant also blocked accumulation of wild type receptor into perinuclear compartments, suggesting subcellular localization may be crucial for signal transduction. A cryptic TRAF-independent NFκB activating region was identified. These mutants define discrete subregions of a novel proline-rich domain that is required for subcellular localization and signal transduction by the LTβR.

The lymphotoxin β (LTβ)1 receptor (LTβR), a member of the tumor necrosis factor receptor (TNFR) superfamily, has emerged as a signaling system required for organization of lymphoid tissue (for reviews, see Refs. 1 and 2). The LTβR binds two distinct but related ligands, the cell surface form of LT (3) and LIGHT (4). Surface LT is composed of two subunits, LTα and LTβ, arranged as a heterotrimer of either LTαβ2 (major form) or LTαβ2 stochiometry (5). The LTβ subunit, a type II transmembrane protein, provides the membrane anchor for the ligand and the specificity for binding the LTβR. The LTα subunit contributes primarily to the conformation of the heterotrimer (6) but can also form homotrimers that bind the two TNF receptors, TNFR1 (55–60 kDa; CD120a) and TNFR2 (80 kDa; CD120b). The second LTβR ligand, LIGHT, a recently identified member of the TNF superfamily (4), forms homotrimers and interacts with another TNFR family member, the herpesvirus entry mediator (HVEM or HveA) (7), which also binds LTα.

Although these ligands show significant cross-receptor specificity, each cytokine-receptor system plays distinct physiologic roles. Based on gene deletion studies, LTβR, but not TNFR, is required for the differentiation of secondary lymph organs, Peyer’s patches, and lymph nodes (8). More recent evidence indicates that progenitor cells crucial for the generation of natural killer cells and dendritic cell compartmentalization require the LTαβ-LTβR system (9–11). LTβR signaling also acts in concert with the TNF/TNFR1 system for the organization of peripheral lymphoid tissue during immune responses (12–15).

Signal transduction by the TNF receptors is initiated by the binding of specific trivalent ligands that induce aggregation of the receptors, which in turn recruit cytosolic proteins involved in the propagation of signals (16). LTβR interacts with TNF receptor-associated factors (TRAFs), a family of zinc RING finger proteins with a C-terminal region that binds directly to the cytoplasmic tail of LTβR and related receptors, such as CD40, CD30, and TNFR80 (17, 18). LTβR binds TRAF2, -3, -4, and -5, but not TRAF6 (19–22). Binding of soluble recombinant LTαβ2 or anti-LTβR antibodies rapidly induces the formation of a stable complex between TRAF3 and LTβR (19). Forced overexpression of these receptors leads to aggregation and activation of signaling pathways independent of ligand, indicating the presence of regulatory mechanisms that normally limit receptor expression or spontaneous aggregation. In cell culture models, signaling through LTβR induces cell death of certain adenocarcinoma tumor cells (23) and gene expression by activation of the p50/p65 form of nuclear factor κB (NF-κB) (19, 24), a transcription factor involved in controlling expression of proinflammatory molecules, including chemokines (25) and integrins (26, 27), and protection of cells from apoptotic death (28). Cell death and NF-κB pathways bifurcate at the level of LTβR-TRAF binding, since TRAF3 mutants block cell death.
signaling but not NF-κB activation (19, 29). TRAF2, -5, and -6 activate the NF-κB pathway by members of the TNFR superfamily, and a common binding site for TRAF2, -3, and -5, the PVQET sequence, has been identified in CD40 (30, 31), but this site is not readily apparent in the LTβR. Identification of the regions in LTβR involved in binding TRAF proteins will aid in understanding the mechanisms of signal propagation. Furthermore, it is unclear whether TRAFs mediate all of the signaling activities of this receptor, because recent findings show that mice with gene deletions in TRAF2, -3, or -5 contain a normal complement of lymph nodes (32–34).

We have identified the structural regions of the LTβR required for TRAF binding and initiation of signaling that activates cell death and gene transcription pathways. A panel of LTβR mutants was characterized that reveal several novel features of LTβR signaling including three discrete regions within a short proline-rich sequence that control TRAF binding and receptor compartmentalization and regulate cell death and NF-κB activation. These mutants should provide useful tools for dissecting the molecular components and pathways involved in physiologic roles dependent on the LTβR.

MATERIALS AND METHODS

Cells and Reagents—Recombinant human TNF (35) and soluble LTα1β2 (36) produced with a truncated version of LTβ lacking the cytosolic and transmembrane domains were provided by Jeffrey Bronning (Biogen, Inc.). Mouse anti-Fas monoclonal antibody (mAb) CH11 (IgM) was obtained from MBL (Nagoya, Japan). M2 and M5 anti-Flag (IgG) mAbs were obtained from Sigma. The anti-c-Myc monoclonal antibody 9E10 (IgG1) was obtained from BABCO. Mouse anti-LTβR antibodies, BDA8 (IgG1) and BK11 (IgG2) were provided by Biogen, Inc. HT29.14s is a clone of the HT29 adenocarcinoma cell line sensitive to the integrity of the c-Myc-LTβR

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structure as described (29). For production of control virus, amphotrophic packaging cell line with the desired pBABE-derived construct (29). All deletion mutants were transfected by the CaPO4 method. Briefly, 250 \( \mu \)g of DNA was introduced into each group. To control for transfection efficiency, a luciferase expression vector was transfected using the common \( \mu \)g primer containing a BglII site, 5′-GACGAGGATCCGGTTCGAGGCTGTAATA-3′. A different \( \mu \)g primer was used for each deletion mutant. Each \( \mu \)g primer included a stop codon and a SsoI site. The 3′-primers used were as follows: Δ418, 5′-GGAGAACCGTGCGTCGTCCGGTGAACATC-3′; Δ418, 5′-GGAAACCGTCTGCACTTAGTGCCTCCGGTGAACA-3′; Δ435, 5′-GGAAACCGTGCACTTAGTGCCTCCGGTGAACA-3′; Δ435, 5′-GGAAACCGTCTGCACTTAGTGCCTCCGGTGAACA-3′; Δ435, 5′-GGAAACCGTCTGCACTTAGTGCCTCCGGTGAACA-3′; Δ435, 5′-GGAAACCGTGCACTTAGTGCCTCCGGTGAACA-3′.

NF-κB activation was measured using a luciferase reporter construct (39). 293T cells seeded at 5 × 10^5 cells/35-mm well were transfected by the CaPO4 method. Briefly, 250 \( \mu \)g of DNA was introduced into each group. To control for transfection efficiency, a luciferase expression vector was transfected using the common \( \mu \)g primer containing a BglII site, 5′-GACGAGGATCCGGTTCGAGGCTGTAATA-3′. A different \( \mu \)g primer was used for each deletion mutant. Each \( \mu \)g primer included a stop codon and a SsoI site. The 3′-primers used were as follows: Δ418, 5′-GGAGAACCGTGCGTCGTCCGGTGAACATC-3′; Δ418, 5′-GGAAACCGTGCGTCGTCCGGTGAACATC-3′; Δ435, 5′-GGAAACCGTGCGTCGTCCGGTGAACATC-3′; Δ435, 5′-GGAAACCGTGCGTCGTCCGGTGAACATC-3′.

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were added to the wells to a final volume of 120 μl/well and incubated in a humidified chamber at room temperature for 1 h. Wells were then washed three times in PBS/BSA/Triton buffer. Fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) in combination with Texas Red-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) were added to the wells to a final volume of 120 μl/well. Slides were incubated in a humidified chamber at room temperature in the dark for 1 h and then washed three times in PBS/BSA/Triton. The slides were mounted in 80% glycerol in PBS, sealed, and kept at 4 °C in the dark for 1–7 days before visualization. Cells were observed with a Bio-Rad MRC-1024 confocal microscope with a krypton/argon ion laser and a 60× Nikon objective. Images were acquired using the LaserSharp operation system and were analyzed and manipulated in Adobe Photoshop. Empty vector-transfected cells or cells stained with normal goat serum or mouse IgG isotype control were used for negative controls. Neither control exhibited background staining. Representative staining patterns were based on counting 200 cells.

**RESULTS**

**Structural Requirements for LTβR Expression and Recruitment of TRAF3**—The cytoplasmic domain of LTβR is 194 amino acids in length and is predicted to belong to the α-β structural superclass (probability = 0.9797) (Fig. 1, A and B). The polypeptide is predicted to emerge from the membrane as a stretch of ~120 residues that fold into three discrete helices, interspersed by β strands, which precedes a proline-rich stretch (38%) of 36 residues (Pro367–Pro403), likely to assume an elongated or kinked pedicle. The pedicle is followed by another β-strand conformation and a fourth helix at the C terminus. Glutathione S-transferase fusion proteins of the LTβR cytoplasmic domain previously indicated that the binding site(s) for TRAF2, -3, and -5 and Hepatitis C virus core protein are all located within residues 338–395, which spans the β-strand and pedicle regions (Ref. 41 and data not shown).

A series of mutants were constructed to determine the subregions of the LTβR involved in TRAF binding and cellular responses. LTβR mutants were made that successively truncate the C terminus through the pedicle and into the β-strand region and incorporate an N-terminal c-Myc epitope tag to distinguish mutant from endogenous receptor (Fig. 1C). HT29.14s cells trans-infected with retrovirus vectors expressing the c-Myc-LTβR deletion mutants revealed striking differences in cell surface expression (Fig. 2). Cells expressing wild type c-Myc-LTβR showed an ~2–3-fold increase over endogenous levels of surface LTβR as estimated from the difference in specific fluorescence staining between anti-c-Myc and anti-LTβR mAb used at saturating levels. Deletion through the C-terminal 36 residues (Δ389) showed no significant change in surface expression; however, the Δ379 mutant expressed little or no staining by anti-c-Myc. Furthermore, ~90% of the endogenous LTβR was also lost from the surface of Δ379 mutant-

**FIG. 1.** Structural features of the LTβR and mutants. A, sequence of the human LTβR cytoplasmic tail. The arrow indicates the region responsible for TRAF and hepatitis C virus core protein interactions. Putative protein kinase C (*) and protein kinase A (***) phosphorylation sites are shown. B, predicted secondary structure. Contour map of the predicted folds of the LTβR cytoplasmic domain as calculated by the Protein Sequence Analysis System. The contour lines show probability increments of 0.1. C, LTβR mutants. Mutants were constructed in a retrovirus expression vector by systematically deleting amino acid residues from the C-terminal cytoplasmic tail of the receptor. Each of these mutants contains an intact extracellular (Ecto) and transmembrane (TM) domain with an N-terminal c-Myc epitope tag. The LTβR deletions are indicated by Δ followed by the initial deleted amino acid.
expressing cells. No evidence was obtained that shedding accounted for the decrease in LTβR expression on the cell surface, indicating that the protein is probably retained intracellular. By contrast, deletion of a further 10 residues (Δ369) led to increased expression on the cell surface of mutant and endogenous LTβR. The Δ369 mutant exhibited an ~5-fold increase in cell surface expression relative to the Δ369 mutant and ~3-fold above wild type receptor.

In unmodified HT29.14s cells, TRAF3 co-immunoprecipitates with the LTβR after brief treatment of cells with ligand (19). In contrast, precipitation with anti-c-Myc revealed that TRAF3 was specifically associated with wild type c-Myc-LTβR (WT), the indicated c-Myc-LTβR deletion mutant, or empty vector (vector). After selection, cells were stained with either mouse anti-c-Myc (9E10, IgG1), anti-LTβR (BKA11, IgG1), or control mouse IgG and detected with phycoerythrin-conjugated goat anti-mouse IgG. Cells were transinfected with the indicated LTβR-expressing vector and stained with control mouse IgG (thin line) or infected with the control vector (thick dark line) or with the indicated LTβR vector (gray thick line) and then stained with either anti-c-Myc (left column) or anti-LTβR (right column). Each histogram represents analysis of 5 × 10⁶ cells.

Fig. 2. Cell surface expression of the c-Myc-LTβR deletion mutants in HT29.14s. HT29.14s cells were infected with retrovirus directing the expression of the wild type c-Myc-LTβR (WT), the indicated c-Myc-LTβR deletion mutant, or empty vector (vector). After selection, cells were stained with either mouse anti-c-Myc (9E10, IgG1), anti-LTβR (BKA11, IgG1), or control mouse IgG and detected with phycoerythrin-conjugated goat anti-mouse IgG. Cells were transinfected with the indicated LTβR-expressing vector and stained with control mouse IgG (thin line) or infected with the control vector (thick dark line) or with the indicated LTβR vector (gray thick line) and then stained with either anti-c-Myc (left column) or anti-LTβR (right column). Each histogram represents analysis of 5 × 10⁶ cells.

molecular mass (~48–55 kDa), indicating that TRAF3 is specifically affected by the Δ379 mutation. The nature of this form of TRAF3 is unknown, but it could represent a proteolytic fragment among other possibilities. The analysis of TRAF3 binding was extended to HEK293 cells, including two additional mutants, Δ396 and Δ389, which showed that TRAF3 binding was specifically lost in Δ389 mutant (Fig. 3B). This result locates the TRAF3 binding site to residues AAPEEGDP.

Expression of LTβR in HEK293 cells monitored by Western blot (Fig. 3C) revealed a single 69-kDa band for wild type
receptor and proportionally smaller forms through mutant Δ403. Mutants Δ396 and Δ389 were resolved as a tight doublet in which the smaller form became predominant in mutants Δ379 and Δ369. This suggests that post-translation modification (e.g., glycosylation) of the receptor was affected by the Δ379 mutation. The Δ359 and Δ345 mutants were not detected by blotting, although expression was readily detected on the cell surface by fluorescence staining, indicating that the overall abundance of these two mutants was decreased.

The loss of endogenous LTβR from the surface of HT29 cells and the coimmunoprecipitation of LTβR and TRAF3 in the absence of ligand prompted us to investigate the subcellular location of these mutants. LTβR and TRAF3 were investigated in the 293T cell line, which does not express detectable cell surface LTβR. Following transfection of the LTβR mutants into 293T cells, fluorescence staining analysis showed a pattern of expression similar to transinfected HT29.14s or HEK293 cells (data not shown). Confocal imaging revealed that the majority of wild type LTβR and mutants through Δ369 accumulated as large clusters in perinuclear compartments (Fig. 4, a–f), and the diminished staining of cells that were fixed but not permeabilized indicated an intracellular location (data not shown). By contrast, Δ359 exhibited a diffuse, primarily surface-staining pattern, indicating that 360NIYIYNGPVI369 is crucial for localization to these vesicles (Fig. 4g). TRAF3 expressed by itself exhibited a diffuse cytoplasmic staining in 293T cells, but when coexpressed with wild type LTβR, it localized to the same perinuclear compartments, a pattern that was not altered by deletion of the LTβR through Δ396 (Fig. 4, h–j). As expected, TRAF3 failed to co-localize with the Δ389 mutant and was dispersed throughout the cytosol; however, LTβR remained in the perinuclear compartment (Fig. 4, k–n). Together, these results indicate that the TRAF3 binding site is defined by the Δ389 mutant, and this region is distinct from the region (Δ359) controlling subcellular compartmentalization of the LTβR. Interestingly, lysosome markers, cathepsin D or LAMP-1, and the endoplasmic reticulum marker AP-1 did not co-localize with the LTβR (data not shown). This result indicates that these LTβR/TRAF3-associated vesicles are unlikely to represent obstructed ER due to overexpression, nor do they appear to be a degradative end point. Further characterization of the subcellular compartments containing LTβR is in progress.

That the Δ379 mutant failed to co-localize or bind directly to TRAF3 in 293T cells but co-immunoprecipitated with TRAF3 in HT29.14s cells indicates that an indirect mechanism allows TRAF3 to associate with the Δ379 mutant. A likely possibility is that Δ379 mutant associates with endogenous LTβR, which is complexed with TRAF3. This predicts that the Δ379 mutant should coimmunoprecipitate in a complex with wild type LTβR and TRAF3. To test if this association occurs, 293T cells were cotransfected with c-Myc-LTβRΔ379, TRAF3-FLAG, and wild type LTβR lacking an epitope tag. As predicted, the lysates subjected to immunoprecipitation with anti-c-Myc showed specific co-immunoprecipitation of TRAF3 by its association with c-Myc-LTβRΔ379 only in the presence of wild type LTβR (Fig. 5A). This association was also visualized by confocal microscopy (Fig. 5B), where TRAF3-FLAG colocalized with Δ379 in

Fig. 4. Co-localization of LTβR and TRAF3. 293T cells were transfected with a total of 2 μg of DNA/35-mm well as follows. a and h, empty vector; b and i, wild type LTβR; c and j, Δ396; d and k, Δ389; e and l, Δ379; f and m, Δ369; g and n, Δ359. Cells were cotransfected with empty vector (panels a–g) or with TRAF3-FLAG (panels h–n). LTβR was stained with goat anti-LTβR and TRAF3-FLAG with mouse anti-FLAG M2. Texas Red-conjugated donkey anti-goat IgG (red) and fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (green) were used to visualize their respective antigens. Yellow indicates colocalization.
the perinuclear compartment only in the presence of wild type LTβR. We further utilized this transfection system to analyze the effect of the Δ359 dominant negative mutant. Confocal microscopy revealed that not only does the presence of Δ359 inhibit TRAF3 from colocalizing with wild type LTβR, but Δ359 also inhibited the accumulation of wild type receptor in the perinuclear compartments.

The Effect of Dominant Positive and Negative Mutants of LTβR on Cell Death Signaling—HT29 cells expressing the c-Myc-LTβR deletion mutants were treated with LTα1β2 or anti-LTβR mAbs, (CBE11), and cell viability was determined after 3 days. In this model, treatment of the cells with IFN-γ is essential for apoptotic cell death induced by LTα1β2, TNF, or Fas (23, 46). Additionally, the anti-LTβR mAb CBE11 added in the soluble phase is normally not directly cytotoxic to HT29.14s cells unless combined with an additional anti-LTβR antibody (5). Treatment with IFN-γ of HT29.14s cells transduced with empty vector resulted in slight growth enhancement when compared with cells in medium, although together with LTα1β2 (1 nM) it induced a 50% decrease in cell viability, whereas LTβR antibody was not cytotoxic (Fig. 6). By contrast, HT29.14s cells expressing wild type c-Myc-LTβR responded to IFN-γ treatment in the absence of LTα1β2 with ~30% loss of cell viability when compared with cells in medium, although together with LTα1β2 (1 nM) it induced a 50% decrease in cell viability, whereas LTβR antibody was not cytotoxic (Fig. 6). By contrast, HT29.14s cells expressing wild type c-Myc-LTβR responded to IFN-γ treatment in the absence of LTα1β2 with ~30% loss of cell viability when compared with cells in medium, although together with LTα1β2 (1 nM) it induced a 50% decrease in cell viability, whereas LTβR antibody was not cytotoxic (Fig. 6). By contrast, HT29.14s cells expressing wild type c-Myc-LTβR responded to IFN-γ treatment in the absence of LTα1β2 with ~30% loss of cell viability when compared with cells in medium, although together with LTα1β2 (1 nM) it induced a 50% decrease in cell viability, whereas LTβR antibody was not cytotoxic (Fig. 6). 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Δ369 mutant displayed a phenotype similar to the cells transduced with empty vector control, suggesting that this mutant is inactive and that the cell death response due to ligand alone occurs via endogenous LTβR. This is supported by the previous finding that Δ369 itself does not bind TRAF3. In contrast to the Δ369 mutant, Δ359 and Δ345 mutants were nonresponsive to LT to p12 (Fig. 6).

The ligand-independent cell death induced by Δ379, together with the finding that this mutant down-regulates endogenous LTβR and indirectly co-immunoprecipitated with TRAF3, defines the behavior predicted for a dominant positive mutant. On the other side, the Δ359 mutant acts as a dominant negative, perhaps due to increased cell surface expression relative to wild type receptor or by blockade of wild type receptor trafficking into perinuclear compartments or association of wild type LTβR with TRAF3.

Ligand-dependent and -independent Activation of p50 and p50/p65 NF-κB Complexes by the LTβR—That NF-κB activation by the LTβR is not inhibited by dominant negative TRAF3 mutants indicates that signaling bifurcates at the level of the receptor (19). The c-Myc-LTβR deletion mutants were examined for their ability to activate NF-κB by electrophoretic mobility shift or NF-κB-dependent reporter assays. HT29.14s cells expressing wild type c-Myc-LTβR displayed constitutive NF-κB binding to the human immunodeficiency virus long terminal repeat NF-κB site, in contrast to empty vector-infected cells, which required treatment with anti-LTβR antibody (Fig. 7A, upper panel). The constitutive κB binding complex completely shifted with antibodies specific for the 50-kDa subunit (p50, NF-κB) but not the 65-kDa subunit of NF-κB (p65, RelA) (Fig. 7B, upper panel). The constitutive p50 complex probably represents dimers of p50 (herein referred to as p50 dimers) but may also form a complex with members of the Rel family other than p65. Constitutive activation of p50 by c-Myc-LTβR was lost in the Δ396, indicating that the region controlling activation of p50 dimers is adjacent to the TRAF binding region. As expected, the Δ379 mutant was active, which further supports the idea that this mutant acts as a dominant positive by activating endogenous receptors. The deletion mutant Δ359 did not stimulate formation of constitutive p50 dimers.

Treatment of empty vector-infected HT29.14s with anti-LTβR mAb resulted in the formation of NF-κB binding complexes (resolved as a tight doublet) (Fig. 7A, middle panel), previously shown to supershift with antibodies to p65 and p50 (19). By contrast, anti-LTβR treatment of cells expressing wild type c-Myc-LTβR revealed an additional κB band (compare lanes with vector and wild type in Fig. 7A, middle panel). Anti-p65 shifted the migration of the upper two complexes, demonstrating the presence of p56 subunit, but had no affect on the fast migrating band (Fig. 7B, lower panel). A nearly complete mobility shift of these bands occurred with anti-p50 antibody (the small fraction of residual band may represent p65 homodimers). This result indicates that both p50/p65 heterodimers and p50 dimers can be activated in HT29.14s cells by LTβR signaling. Note that treatment of control HT29.14s cells (empty vector alone) with anti-LTβR did not activate the p50 within this short time frame. Furthermore, the presence of p50 in anti-LTβR-activated cells was dependent on the same region (Δ396) of the receptor as unstimulated bands (Fig. 7A, upper panel). Together, these results indicate that the pattern of NF-κB bands in HT29.14s cells stimulated with anti-LTβR is a composite of the rapidly induced p65/p50 dimers and constitutively formed p50 dimers. Analysis of the κB bands in cells after treatment for 15 min with anti-LTβR showed that formation of p50/p65 heterodimer was inhibited only by the Δ359, further establishing this mutant as a dominant negative that inactivates the function of the endogenous LTβR (Fig. 7B, middle panel).

As expected, none of these LTβR mutations ablated the formation of p50/p65 complexes induced by TNF (Fig. 7A, lower panel), and TNF had no detectable effect on the presence of the p50 dimers. However, TNF treatment of HT29.14s cells expressing the wild type c-Myc-LTβR resulted in an enhanced activation of the p50/p65 NF-κB complex (~4-fold). TNF treatment of the remaining c-Myc-LTβR deletion mutants, including the dominant negative mutant c-Myc-LTβRΔ359, also resulted in enhanced activation of the p50/p65 NF-κB heterodimer. These data suggest that TNF-induced activation of NF-κB may cooperate with the LTβR. Even the Δ359 mutant, which is incapable of activating NF-κB in response to LTβR ligation, can contribute to the enhancement of NF-κB activation by TNF, suggesting that additional sequences in the N-terminal proximal region are responsible for this NF-κB enhancing activity.

The relatively complex pattern of NF-κB activation in HT29.14s cells prompted us to examine the effect of these mutants in 293T cells. Expression of wild type receptor and mutants Δ418 through Δ396 conferred NF-κB activation as measured by NF-κB-dependent luciferase reporter (Fig. 7C). That Δ396 was functional in this assay but not in HT29.14s is surprising. It is possible that overexpression in 293T can compensate for this mutation. The Δ389 mutant, which deletes the TRAF3 recruitment domain, was inactive. However, significant activation of NF-κB occurred with further truncation of the LTβR including Δ379, Δ369, and Δ359, although with a less robust signal, but was lost with the Δ345 mutant. The lack of a dominant negative effect of Δ359 in this system, as well as the absence of endogenous LTβR for the Δ379 to act through, implicates a TRAF-independent mechanism of NF-κB activation by the LTβR. This result indicates the presence of a cryptic NF-κB activation site that is normally inhibited by the sequence defined by the Δ389 mutant and confirms our suspicion of an additional region that can activate NF-κB. The components involved in this NF-κB pathway are currently being explored.

**Discussion**

The LTβR deletion mutants analyzed here define a subregion of the LTβR cytoplasmic domain between Leu349 and His353 that functions as a key structural element for receptor compartmentalization and signal transduction (Fig. 8). This sequence encompasses a proline-rich region that is predicted to have an elongated or kinked conformation. Several of the deletion mutants were informative in that they defined discrete sequences with distinct functions including subcellular compartmentalization, TRAF binding, and regulation of cell death and activation of NF-κB. The use of distinct cellular models was invaluable in realizing the effects of these mutations. One significant difference is that HT29.14s cells express endogenous LTβR, whereas 293T cells do not, and this difference clearly affected the behavior of the Δ379 and Δ359 mutants.

Two distinct binding motifs for TRAF2, -3, and -5 are found in TNFR family members: FXQET in CD40 (FXQX(T/S) consensus motif in CD27, CD30, 4-1BB, OX40, and Epstein-Barr virus oncoprotein LMP-1) and SKEEC in TNFR2 (a similar motif is also in herpesvirus entry mediator and CD30) (18). Recent crystallographic studies of TRAF2 in complex with TRAF binding peptide from either CD40 (42) or TNFR2 (43) reveal the residues in these peptides that contact TRAF2 are distinct, although the binding affinity and conformation of the peptides are quite similar. The TRAF3 binding region in the LTβR was localized to the sequence 389PEEGDP, which is distinct from both CD40 and TNFR2 motifs. In preliminary results, mutation of both glutamate residues (Glu391-Glu392) in
for TNFR1 (44, 45). In this regard, expression of wild type LTβR in HT29.14s cells, although only modestly increasing surface expression, enhanced the responsiveness to cell death, recruitment of TRAF3, and NF-κB activation. These findings are consistent with the idea that exceeding a certain threshold of receptor density increases the probability that receptors will spontaneously aggregate and initiate limited signal transduction in the absence of ligand. Indeed, modest overexpression was sufficient for activation of p50 complexes of NF-κB but not the p50/p65 complex. Furthermore, HT29.14s cells still required ligand to induce cell death, indicating that additional mechanism(s) prevented full receptor activation, which implicates the region spanning 379PATPEPPYPI as a critical regulatory sequence.

The enhanced responsiveness of HT29.14s cells expressing wild type LTβR or the other mutants (except for Δ359) and the insensitivity of 293T cells to death induced by LTβR precluded an unambiguous test to define the role of the TRAF binding site in the cell death pathway. However, previous results with dominant negative forms of TRAF3 indicate that recruitment and oligomerization of TRAF3 is important to specifically activate the LTβR death pathway (29, 46). How TRAF3 propagates the signal to the death pathway remains to be elucidated.

The Δ359 mutant functioned as a dominant negative mutant in HT29.14s cells that suppressed the signaling action of endogenous LTβR for cell death and NF-κB activation. This mutant was expressed at increased levels on the cell surface, potentially mediating its dominant negative effect as a decoy receptor (i.e. retaining ligand binding but lacking signaling capacity). An alternative or contributing mechanism is suggested from confocal microscopy, which showed that the Δ359 mutant effectively blocked accumulation of wild type LTβR into the perinuclear compartments. This result implies that entry into this compartment may be necessary for signal transduction. That both dominant positive and negative mutants can interact with wild type receptors indicates that LTβR contains a self-association domain that is membrane-proximal to Δ359. Recent studies by Wu et al. (46) using large deletions mapped a self-association domain to amino acids 324–377, consistent with our results.

LTβR initiates transcription by activating NF-κB that is dependent on TRAF5 or TRAF2. However, recent evidence
from gene knockouts challenges this idea. TRAF2-, TRAF3-, or TRAF5-deficient mice exhibit secondary lymphoid tissue development, a phenotype that is observed by deletion of LTβR (8). Although TRAF6 does not appear to bind LTβR, mice deficient in TRAF6 fail to develop lymph nodes, a phenotype that is thought to be linked to the osteoclast differentiation factor (OPGL/RANK) pathway (47). The alymphoplasia (in TRAF6 fail to develop lymph nodes, a phenotype that is

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Discrete Signaling Regions in the Lymphotoxin-β Receptor for Tumor Necrosis Factor Receptor-associated Factor Binding, Subcellular Localization, and Activation of Cell Death and NF-κB Pathways

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