The Cytoplasmic Domain of the Lymphotoxin-β Receptor Mediates Cell Death in HeLa Cells*

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Activation of lymphotoxin-β receptor (LT-βR) by conjugation with heterotrimeric lymphotoxin, LT-α1/β2, or by cross-linking with anti-LT-βR antibodies can trigger apoptosis. We have observed that overexpression of either LT-βR or the cytoplasmic domain of LT-βR (LT-βR(CD)) also induces apoptosis, which may be attributed to the tendency of LT-βR(CD) to self-associate. The self-association domain of LT-βR(CD) was mapped to amino acids 324–377, a region of the protein that is also essential for LT-βR-induced apoptosis. Furthermore, we have shown that LT-βR(CD)-induced apoptosis could be inhibited by a TRAF3 dominant negative mutant and by the caspase inhibitors Z-VAD-FMK, DEVD-FMK, and CrmA. The ligand-independent apoptosis induced by LT-βR(CD) will help us to further dissect LT-βR signaling pathway.

Lymphotoxin-β receptor (LT-βR)† is a member of the tumor necrosis factor receptor (TNFR) superfamily and is expressed on the surface of most of cell types, including cells of epithelial and myeloid lineages but not on T and B lymphocytes (1, 2). LT-βR can bind specifically to two ligands: the membrane form of lymphotoxin, LT-α1/β2, (3, 4); and LIGHT, a recently identified member of TNF superfamily (5, 6). LT-βR has been speculated to play an essential role in the development of lymphoid organs. In LT-α knock-out mice, the development of lymphoid organs is prevented (7). Studies involving LT-β knock-out mice have shown impairment of lymph node development and loss of splenic architecture (8). Similar results were observed when the soluble LT-β receptor-immunoglobulin Fc chimera fusion protein was introduced into the embryonic circulation by injecting pregnant mice (9). Direct evidence to demonstrate the role of LT-βR in lymphoid organ development comes from the fact that LT-βR deficient mice lack Peyer’s patches, colon-associated lymphoid tissues, and all lymph nodes (10). Moreover, injection of the agonist anti-LT-βR monoclonal antibody into the uteri of pregnant LT-α knock-out mice has been shown to result in the genesis of lymph nodes in their progeny (11). In addition to its role in lymphoid organ development, stimulation of LT-βR on certain cell lines by LT-α1/β2 or anti-LT-βR antibodies can induce cell death (12), chemokine secretion (13), and activation of nuclear factor κB (NF-κB) (14). Thus, LT-βR may also have important biological functions in the mature individuals.

The cytoplasmic domain of LT-βR, like other members of the TNF receptor family, does not contain consensus sequences characteristic of enzymatic activity. Therefore, signaling is thought to be mediated by the proteins interacting with LT-βR. Two serine/threonine protein kinases, p50 and p80, have been shown to associate with LT-βR(CD) specifically (15), but the function of p50 and p80 in the LT-βR signaling pathway is still the subject of intensive study. Moreover, two members of the TNF receptor-associated factor (TRAF) family, TRAF3 and TRAF5, were found to associate with LT-βR (16, 17). Further study has indicated that TRAF3 plays an important role in mediating LT-βR-induced apoptosis (18, 19), whereas TRAF5 has been shown to be involved in the activation of NF-κB (17). On the other hand, several members of TNFR superfamily (such as TNFRI, Fas, DR3, DR4, and DR5) contain a common motif, the death domain, in their cytoplasmic region (19–24). These “death receptors” interact with other death domain-containing proteins, such as TRADD (25), MORT1/FADD (26), RIP (27), and RAIDD (28), via their death domains. MORT1/FADD and RAIDD can in turn interact with MACH1/FLICE (caspase-8) and caspase-2, respectively (28–30), and thus initiate the activation of caspase cascades to execute apoptosis (31). LT-βR(CD) does not contain a death domain, but signaling through LT-βR can also induce apoptosis (12). It will be interesting to map the region of LT-βR responsible for its cytotoxic effect and to determine whether LT-βR mediates apoptosis via the activation of caspase cascades.

Both TNFRI and TNFRII can induce cell death when bound by TNF. It has also been reported that clustering of TNF receptors due to interaction either with trivalent TNF or with an agonist antibody is a crucial step for subsequent intracellular signaling (32, 33). Because the cytoplasmic domain of TNFRI can self-assemble through its death domain (34, 35), overexpression of TNFRI or of its cytoplasmic domain alone can also induce receptor clustering resulting in the activation of downstream signaling pathways (34, 36). In contrast, TNFRII has no death domain and shows no tendency to self-associate, nor does a high level of TNFRII expression result in spontaneous signaling (34, 37). In this study, we have shown that LT-βR(CD) is capable of self-association, despite the absence of a death domain. Moreover, overexpression of LT-βR or LT-βR(CD) was sufficient to trigger apoptosis without the need for ligand conjugation. The cytotoxic effect mediated by LT-βR(CD) relies on the presence of its self-association domain,
suggesting that this domain is critical in the LT-βR signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 10% (v/v) fetal bovine serum, in a 37 °C incubator containing 5% (v/v) CO2.

Plasmid Construction—A cDNA fragment encoding the full-length LT-βR was amplified by reverse transcription-polymerase chain reaction using a HepG2-derived cDNA template and the primers 5′-CGGGATCCATGTCGCTCTGCTTGCCAC-3′ (sense) and 5′-CCGGATCTCCATGCTGATGATAATTGG-3′ (antisense). The DNA fragment containing the human LT-βR cytoplasmic domain was amplified by polymerase chain reaction using the primers 5′-GGAAATCTCAAGGCACCCCTTCTCTCTGC-3′ (sense) and 5′-GGAAATCTCCATGCTGATGATAATTGG-3′ (antisense). LT-βR(1-214) (16), was amplified by polymerase chain reaction and subcloned into the pFLAG-CMV2 vector (Eastman Kodak, Co.) in frame with the FLAG tag at 5′ end. The dominant negative mutant of TRAF3, TRAF3(367–568) (16), was ampicillin-selected. For protein-protein interaction assays, plasmids were used to transform Saccharomyces cerevisiae, respectively. For protein-protein interaction assays, plasmids were used to transform Saccharomyces cerevisiae strain Y190. Positive clones were selected by prototrophy for histidine and tested by filter assays for glutathione-agarose beads or with anti-LT-βR antibodies to protein A beads (lane 5). All these samples and HeLa cell extracts (from 2 x 10^6 cells) were fractionated on 7.5% (w/v) SDS-polyacrylamide gels, and Western blot analysis was carried out using peroxidase-conjugated avidin to detect biotinylated proteins. Molecular mass standards (in kDa) are marked on the left, and the position of full-length LT-βR is indicated by an arrow.

fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml neomycin, 100 μg/ml hygromycin, and 2 μg/ml tetracycline. To induce the expression of LT-βR(CD), transfectants were incubated in the above medium without tetracycline.

MITT Test—The survival rate of cells was determined by an MTT test. Briefly, cells were seeded in 96-well flat bottom plates at a density of 3 x 10^3 cells/0.1 ml. After the indicated time period, 10 μl of 5 mg/ml MTT/well was added, and the cells were incubated at 37 °C for 4 h. The cells were then lysed by the addition of 100 μl of 10% SDS in 10 mM HCl per well and incubation at 37 °C for 24 h. The optical density of each sample was determined by measuring the absorbance at 570 nm versus 650 nm using an enzyme-linked immunosorbent assay reader (TECAN, RainBow).

Immunofluorescence Microscopy—Cells were fixed with 1% paraformaldehyde in PBS at room temperature for 20 min and then permeabilized with acetone at −20 °C for 3 min. Cells were then incubated with anti-FLAG monoclonal antibody (5 μg/ml) at room temperature for 1 h, followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse IgG at room temperature for 1 h after washing with PBS three times. Cells were then examined with a MRC600 scanning confocal microscope (Bio-Rad). All the antibodies were diluted in 1% bovine serum albumin/PBS.

RESULTS

Mapping of the LT-βR Self-association Domain—In a yeast two-hybrid screen using LT-βR(CD) as a bait, Chen et al. (39) found that most of the positive clones isolated from a human liver cDNA library corresponded to LT-βR. This was indicative of a tendency to self-associate. To confirm the ability of LT-βR to self-associate, we tested whether a GST-LT-βR(CD) fusion protein is capable of interacting with endogenous LT-βR from HeLa cells. HeLa cells were surface biotinylated as described under “Experimental Procedures.” A GST pull-down assay was carried out, and precipitated proteins were detected with avidin. We found that a 70-kDa protein was coimmunoprecipitated with GST-LT-βR(CD) (Fig. 1, lane 3) but not with GST under the same conditions (Fig. 1, lane 2). Furthermore, the 70-kDa protein associated with GST-LT-βR(CD) could be precleared from...
observations above, we concluded that LT-βR associations are confident that the 70-kDa protein is endogenous LT-βR, the pAS2–1-LT-βR(pAS2–1-LT-βR) protein was transfected with various combinations of these expression constructs. Clones phototrophic for histidine were subjected to filter assays. Blue color indicated the presence of β-galactosidase activity.

| Protein fused to GAL4 domain | DNA binding | Activation | Filter color |
|-----------------------------|-------------|------------|-------------|
| pAS2–1 vector               | pACT2 vector | White      |
| pAS2–1–LT-βR(CD)            | pACT2-LT-βR(CD) | Blue |
| pAS2–1–LT-βR(CD)(aa234–377) | pACT2-LT-βR(CD) | Blue |
| pAS2–1–LT-βR(CD)(aa234–324) | pACT2-LT-βR(CD) | White |
| pAS2–1–LT-βR(CD)(Δ234–377)  | pACT2-LT-βR(CD) | Blue |
| pAS2–1–LT-βR(CD)            | pACT2 vector  | White |

HeLa cell extracts were stained with Hoechst 33342 (data not shown). In contrast, cells transfected with the pFLAG vector alone did not show nuclear condensation (Fig. 2C). The proportion of cells expressing LT-βR or LT-βR(CD) that underwent apoptosis were about 76 and 74%, respectively, whereas this occurred in only ~8% of control cells (Fig. 2D). From this observation, it is clear that overexpression of LT-βR or LT-βR(CD) can result in cell death, without the need for ligand binding or receptor cross-linking by an agonist antibody. Similar results were obtained in the human adenocarcinoma cell line HT29 overexpressing LT-βR or LT-βR(CD) (data not shown).

To further confirm this observation, we used the Tet-off-inducible system to allow the expression of LT-βR(CD) to be turned on when tetracycline was removed from culture medium. In Tet-off HeLa cells stably transfected with pTRE-LT-βR(CD), LT-βR(CD) protein became detectable at day 3 after induction (data not shown). Significant cytotoxicity was observed when tetracycline was removed (Fig. 3A, right panel), whereas cells did not undergo apoptosis in the presence of tetracycline (Fig. 3A, left panel). By staining the surviving cells with MTT, we found that cell death became apparent at day 4 after removal of tetracycline (Fig. 3B). Based on the observations above, we concluded that expression of LT-βR(CD) alone is sufficient to induce apoptosis.

**Characterization of the Apoptotic Pathway Initiated by LT-βR(CD)—** It has been shown that the dominant negative TRAF3 mutant, TRAF3(367–568), can inhibit cell death triggered by LT-α1/β2 or anti-LT-βR antibodies (16). Therefore, we asked whether the TRAF3 mutant can also affect LT-βR(CD)-induced apoptosis. TRAF3(367–568) was cotransfected with LT-βR(CD) into HeLa cells. We found that TRAF3(367–568) provided partial protection from the cytotoxic effect of LT-βR(CD) (Fig. 4A), suggesting that TRAF3 is involved in LT-βR(CD)-induced apoptosis.

Signaling through TNFRI and Fas can initiate caspase cascades to execute apoptosis (31). To examine whether LT-βR(CD) can also activate caspases, the effects of Z-VAD-FMK (a broad spectrum caspase inhibitor), DEVD-FMK (a CPP32 inhibitor), and the CamA of cowpox virus on LT-βR(CD)-induced apoptosis were tested. As shown in Fig. 4B, the cytotoxicity induced by LT-βR(CD) was blocked in cells coexpressing...
Mapping of the Apoptotic Domain of LT-βR—Because there is no death domain in the cytoplasmic region of LT-βR, we wished to identify the sequence responsible for LT-βR(CD)-induced apoptosis. As shown in Fig. 5, apoptosis was observed in cells transfected with either LT-βR(CD) or the deletion mutant LT-βR(CD)(aa234–377). In contrast, the deletion mutants LT-βR(CD)(aa234–324) and LT-βR(CD)(aa234–324) and LT-βR(CD)(aa234–377) had no effect on cell viability. These results provided direct evidence that amino acids 324–377 are essential for LT-βR(CD)-induced apoptosis. This is also the region required for LT-βR self-association (Table I).

Localization of the LT-βR Deletion Mutants—Loss of the extracellular and transmembrane domains would be expected to result in LT-βR(CD) in the cytoplasm of cells. However, LT-βR(CD) was shown to associate with endogenous full-length LT-βR, which is a transmembrane protein (Fig. 1). Therefore, we wished to clarify the localization of the LT-βR(CD). HeLa cells transiently transfected with LT-βR(CD) or its deletion mutants were processed for immunostaining (Fig. 6). Interestingly, we found that LT-βR(CD) and LT-βR(CD)(aa234–377) were localized in the proximity of plasma membrane (Fig. 6, A and B), whereas deletion mutants lacking the self-association domain (LT-βR(CD)(aa234–324) and LT-βR(CD)(aa324–377)) were predominantly localized in the cytoplasm (Fig. 6, C and D). The membrane juxtaposition of LT-βR(CD) was also observed in Tet-off HeLa cells stably transfected with LT-βR(CD) (data not shown). Considering that LT-βR(CD) and LT-βR(CD)(aa234–377) contain the self-association domain and have the ability to self-associate (Fig. 1 and Table I), it is conceivable that they might be directed to the inner side of plasma membrane through their interactions with endogenous LT-βR. This observation suggests that the self-association domains are able to interact in vivo.

FIG. 4. Effects of a TRAF3 dominant negative mutant and caspase inhibitors on LT-βR(CD)-induced apoptosis. A, HeLa cells were cotransfected with pFLAG-LT-βR(CD) and pCMV-lacZ, in conjunction with TRAF3(367–568) in a ratio of 7:1:7. The amounts of total transfected DNA were equalized with control vector. Apoptosis assays were performed as described in the legend to Fig. 2. B, HeLa cells were cotransfected with pFLAG-LT-βR(CD) and pCMV-lacZ at a ratio of 7:1 or with pFLAG-LT-βR(CD), pCMV-lacZ, and CrmA at a ratio of 7:1:5. The cells cotransfected with pFLAG-LT-βR(CD) and pCMV-lacZ were incubated either with the caspase peptide inhibitors Z-VAD-FMK (20 μM) or DEVD-FMK (150 μM) or with dimethyl sulfoxide (DMSO) (0.1%) as a control.

DISCUSSION

The cytoplasmic domains of TNFRI and Fas have been shown to be able to self-associate via their death domains. This self-association can prompt signaling events, giving rise to TNF and Fas effects (34, 35). In contrast, the cytoplasmic region of TNFRII has no death domain and shows no tendency to self-associate, nor does overexpression of this receptor induce cell death (34, 37). Like TNFRII, LT-βR does not contain a death domain. However, studies using the yeast two-hybrid and GST-pull down systems have clearly demonstrated the self-association tendency of LT-βR(CD) (Ref. 39 and the present report). In addition, we have observed that overexpression of LT-βR or LT-βR(CD) is sufficient to trigger apoptosis, without the need to cross-link LT-βR with ligand or an agonist antibody. It has

Fig. 3. Viability of Tet-off HeLa cells transfected with LT-βR(CD). The tetracycline-controlled construct encoding the LT-βR(CD) was stably transfected into Tet-off HeLa cells that express the tetracycline-controlled transactivator constitutively (CLONTECH). A, cells were examined under a phase contrast microscope in the absence and presence of tetracycline. Photographs were taken at day 6 after tetracycline was removed. B, the viability of cells was determined by MTT tests at the times indicated. The percentage of survival cells in the absence of tetracycline was compared with the cell population in the presence of tetracycline at various times.
been shown that a dominant negative mutant of TRAF3, TRAF3(367–568), can inhibit apoptosis induced by LT-α1/β2 or anti-LT-βR antibodies (16). Likewise, our results showed the dominant negative effect of TRAF3(367–568) on LT-βR(CD)-induced apoptosis. This result suggests that apoptotic signaling mediated by LT-βR(CD) may share the same pathway as that triggered by ligand conjugation. Furthermore, activation of NF-κB was also observed in cells overexpressing LT-βR(CD). This is in accordance with previous reports that stimulation of LT-βR by recombinant LT-α1/β2 or anti-LT-βR antibodies can transduce signals not only for apoptosis (12) but also for NF-κB activation (14). Based on the observations above, overexpression of LT-βR(CD) seems to be able to activate downstream signaling events equivalent to those induced by LT-α1/β2 or anti-LT-βR antibodies.

It has been shown that apoptotic signaling mediated by TNFRI or Fas occurs via interaction with other death domain-containing proteins, which can initiate caspase cascades to execute apoptosis (31). Here, we demonstrated that LT-βR(CD)-induced apoptosis was inhibited by several caspase inhibitors: CrmA, Z-VAD-FMK, and DEVD-FMK, suggesting that the activation of CPP32 or ICE-related caspases might be involved in the apoptotic signaling of LT-βR. Because LT-βR does not contain a death domain, it will be intriguing to discover how LT-βR initiates the caspase pathway. This question might be answered by examining whether TRAF3 or other LT-βR(CD)-associated proteins could recruit and activate caspase(s).

LT-α1/β2 or anti-LT-βR antibodies cannot trigger apoptosis without the presence of IFN-γ (12). However, LT-βR(CD)-induced apoptosis does not require the presence of IFN-γ, nor did IFN-γ result in a synergistic enhancement of LT-βR(CD) cytotoxicity (data not shown). The mechanism of IFN-γ action in apoptosis induced by LT-βR is still unclear. Recently, IFN-γ was found to modulate cell death by inducing several apoptosis-related genes, including the TNFR family members, TNFRI and Fas; a bcl-2 family member, bak; and the caspase family members, ICE, CPP32, and FLICE (40). Nevertheless, IFN-γ does not up-regulate LT-βR expression on HT-29 (12) and HeLa (data not shown) cell lines. It is possible that IFN-γ might modulate LT-βR-induced cell death by regulating caspases involved in the cytotoxic effect. However, the amounts of LT-βR(CD) expressed by transfected cells might be sufficient to initiate caspase cascades to execute apoptosis, such that IFN-γ does not further enhance the cytotoxic effect. This speculation is supported by the fact that LT-βR(CD)-induced apoptosis can be inhibited by several caspase inhibitors, suggesting that the activation of caspases is important in this apoptotic pathway.

Because there are no consensus sequences for membrane anchorage in the FLAG tag or LT-βR(CD), the FLAG-LT-βR(CD) fusion protein would be expected to express as a cytoplasmic protein. However, the FLAG-LT-βR(CD) was shown to be localized in the proximity of plasma membrane. Interestingly, we also observed that membrane juxtaposition of LT-βR(CD) and its deletion mutants correlates with their ability to self-associate and to induce apoptosis. Because the self-association domain is required for targeting of LT-βR(CD) to the plasma membrane (Fig. 6), we speculate that membrane juxtaposition of LT-βR(CD) might be due to its association with endogenous LT-βR (Fig. 1), which may therefore induce receptor clustering to activate apoptotic signaling. Nevertheless, our result does not rule out the possibility that LT-βR(CD) can be linked to the membrane by other proteins, either located on the inner side of cell membrane or associated with endogenous LT-βR.

Previous studies have shown that the death domain of TN-
FRI has a strong tendency to self-associate and interact with other death domain-containing proteins and plays an obliga-
tively role in signaling cell death. In the present report, self-
association of LT-βR(CD) was mapped to amino acids 324–377,
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