Serine/Threonine Kinase Activity Associated with the Cytoplasmic Domain of the Lymphotoxin-β Receptor in HepG2 Cells

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The lymphotoxin-β receptor (LT-βR) has been shown to be the receptor for the membrane-bound lymphotoxin heterotrimers LTα1/β2 and LTα2/β1. The extracellular domain of LT-βR shows extensive similarity with members of the tumor necrosis factor receptor family, while its cytoplasmic domain is distinct and lacks any inherent enzymatic activity. This suggests that the association of LT-βR with other molecules might be important for signal transduction. Here we demonstrate the association of a fusion protein, comprising glutathione S-transferase and the cytoplasmic domain of LT-βR (GST-LT-βR(CD)), with several proteins in the size range 29–80 kDa from HepG2 cell lysates. We present evidence that two of these proteins are serine/threonine kinases, which associate with amino acids 324–377 of the cytoplasmic domain of LT-βR and phosphorylate this receptor. The characteristics of these novel kinases indicate that they are distinct from the previously described tumor necrosis factor receptor-associated kinases. This suggests the presence of novel signal transduction pathway(s) for LT-βR.

Tumor necrosis factor (TNF)α is a pleiotropic factor which induces an extraordinarily wide variety of biological responses, mainly related to immune regulation and inflammation (1, 2). Two structurally-related molecules, TNFα and lymphotoxin (LT)α (also known as TNFβ), are encoded by two genes located just 1 kilobase apart in the class III region of the human major histocompatibility complex (3). Both TNFα and LTα can bind to tumor necrosis factor receptors type I and type II (TNFRI and TNFRII) to mediate similar biological effects. Recently, a member of the TNF/LT family known as LTβ, which is encoded by a gene lying 4 kilobases centromeric to the TNFα/LTα genes, was found to be expressed on the surface of natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and activated T and B lymphocytes (3–5). In contrast to the homotrimeric structures of TNFα (which occurs as membrane-bound and secreted forms) and LTα (which is secreted), LTβ is a 33-kDa transmembrane protein, which associates with LTα and anchors it to the cell surface. Stoichiometrically, membrane lymphotoxin is a heterotrimer and is composed of either LTα1/LTβ2 (major form) or LTα2/LTβ1 (minor form) (6). The LTα2/LTβ1 heterotrimer has been shown to bind to TNFRI, TNFRII, and LT-βR, while the LTα1/LTβ2 heterotrimer binds only to LT-βR (also known as TNFRIII, or TNFR-related protein (TNFRrp)) (6–8).

In contrast to TNFRI and TNFRII, the functions of LT-βR are still not well understood. In LTα knock-out mice, which lack the membrane form of lymphotoxin, normal development of peripheral lymph nodes is ablated (9, 10). Since this phenotype is not observed in either TNFRI or TNFRII knockout mice (11–13), it has been speculated that signaling through LT-βR might be involved in the development of lymphoid organs. Furthermore, stimulation of LT-βR by immobilized anti-LT-βR monoclonal antibodies has been shown to trigger activation of NF-κB in HT-29 (human adenocarcinoma) and WI38 (human lung fibroblast) cells (14) and signaling through LT-βR in conjunction with interferon-γ can induce cell death through apoptosis in some adenocarcinoma cell lines (15). These studies provide evidence that LT-βR can transduce signals that trigger both cell proliferation and apoptosis. The cytoplasmic domain of LT-βR, like other members of the TNF receptor family, does not contain consensus sequences characteristic of known kinases or any motifs associated with signal transduction. Therefore, kinases or other proteins associated with the cytoplasmic domain of LT-βR are likely to be involved in the signal transduction pathway. Recently, two members of the TNF receptor-associated factor (TRAF) family, TRAF3 and TRAF5, were found to associate with LT-βR (16, 17). There is evidence that TRAF5 is involved in the activation of NF-κB (17), but the role of TRAF3 in the LT-βR signaling pathway has not been reported.

While members of the Janus kinase (JAK) family have been shown to play a key role in signal transduction via many cytokine receptors (18–20), the nature of the kinases involved in the signal transduction pathways of the TNF receptor family has only recently been investigated. Several groups have shown that the cytoplasmic domains of both TNFRI and TNFRII can associate with serine/threonine kinases. Darnay et al. (21, 22) have identified serine/threonine kinases that specifically associate with fusion proteins comprising glutathione S-transferase and the cytoplasmic domain of TNFRI (GST-p60) and TNFRII (GST-p80). Furthermore, the TNFRI-associated kinase (p60-TRAK) was shown to bind residues 344–397 of the LT-βR, like other members of the TNF receptor family, does not contain consensus sequences characteristic of known kinases or any motifs associated with signal transduction. Therefore, kinases or other proteins associated with the cytoplasmic domain of LT-βR are likely to be involved in the signal transduction pathway. Recently, two members of the TNF receptor-associated factor (TRAF) family, TRAF3 and TRAF5, were found to associate with LT-βR (16, 17). There is evidence that TRAF5 is involved in the activation of NF-κB (17), but the role of TRAF3 in the LT-βR signaling pathway has not been reported.

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TNFRI-associated protein, RIP, has been shown to be a serine/threonine kinase, which contains a kinase domain in its N terminus and a death domain in its C terminus. The TNF-dependent recruitment of RIP to the TNFRI signal complex is mediated by a TNFRI-associated death domain-containing protein (TRADD) and has been shown to induce apoptosis and NF-κB activation following receptor triggering (25). However, it is unclear whether or not RIP can phosphorylate TNFRI. The TNFRI-associated kinase has been identified as casein kinase-1 (CK-1) (26) and CKI-7, an inhibitor of CK-1, has been shown to inhibit the phosphorylation of TNFRI and to potentiate TNF-induced apoptosis. This evidence suggests that CK-1 is shown to be involved in signal transduction through TNFRI. Based on the information above, we wished to determine whether LT-βR is associated with kinase activity. Here we report the identification of two serine/threonine kinases specifically associated with LT-βR and demonstrate that these can phosphorylate LT-βR, but not TNFRI. These novel kinases are likely to be important in LT-βR signal transduction pathway(s).

EXPERIMENTAL PROCEDURES

Cell Culture and Radioisotope Labeling—Mammalian cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% (v/v) fetal bovine serum, in a 37 °C incubator containing 5% (v/v) CO2. For radioisotope labeling, cells were washed twice with Hank’s balanced salt solution (8 g/liter NaCl, 0.4 g/liter KCl, 0.2 g/liter MgSO4·7H2O, 60 mg/liter Na2HPO4, 60 mg/liter KH2PO4, 1 g/liter glucose, 140 mg/liter CaCl2), then incubated in cytosine/methionine-free medium containing 10% (v/v) dialyzed fetal calf serum (Life Technologies, Inc.) for 1 h, followed by addition of 50 μCi/ml [35S]cytochrome (NEN Research Products) and incubation at 37 °C for 6 h before harvesting.

Construction of GST Expression Vectors—To construct pGST-LT-βR(CD), a 540-base pair PstI/HindIII cDNA fragment (nucleotides 767–1305) encoding almost the full length of the human LT-βR cytoplasmic domain was amplified by reverse transcription-PCR using a HepG2-derived cDNA template and the primers 5'-CGGATCCATGCTCCT-GCTTGGGGCCAC-3' (sense) and 5'-GGCAGATCTGGGGGAGTG-CCTATGG-3' (antisense). The PCR product was end-filled, cloned into Smal-cut pGEX-2T vector, and sequenced to confirm that the pGST-LT-βR(CD) construct contained no deleterious PCR errors. Deletion mutants were generated by restriction enzyme digestion and subsequent religation of pGST-LT-βR(CD).

Expression and Purification of GST Fusion Proteins—The expression and purification of GST-fusion proteins were carried out as described by Darnay et al. (21) with the following modifications. JM109 bacteria containing the pGST-LT-βR(CD) were grown to A600 = 0.2 at 37 °C before adding isopropyl-1-thio-β-D-galactopyranoside to 0.1 μm. After 3 h of induction, bacteria were lysed with 1 ml A20 (20 ml Tris, 0.2 M NaCl, 10% (v/v) glycerol, 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1% (v/v) 2-mercaptoethanol, 0.5% (v/v) Nonidet P-40) containing 100 μg/ml lysozyme. After brief sonication, the bacterial lysate was centrifuged at 3500 × g, for 5 min at 4 °C to remove insoluble debris. The supernatant was incubated with 50% (v/v) glutathione-agarose beads (Sigma) at 4 °C for 30 min on a rotatory shaker. The slurry was then washed five times with buffer A, and stored as a 50% (v/v) slurry at 4 °C.

In Vitro Binding of GST-LT-βR(CD) with HepG2 Cell Lysates—The in vitro binding assay was modified from that described by Darnay et al. (21). Briefly, 1 × 109 [35S]cytochrome/methionine-labeled HepG2 cells were resuspended in 1 ml of lysis buffer (20 mM Tris, pH 7.7, 0.5% (v/v) Nonidet P-40, 200 mM NaCl, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1% (v/v) 2-mercaptoethanol) for 1 h at 4 °C, followed by centrifugation at 9000 × g for 10 min to remove cell debris. Before incubation with the GST-LT-βR(CD) fusion protein, the supernatant was precleared with 15 μg of GST and 50 μl of 50% (v/v) glutathione-agarose beads for 2 h at 4 °C, followed by centrifugation at 800 × g for 3 min at 4 °C. The supernatant was then transferred to a fresh tube and mixed for 2 h with 10 μg of GST-LT-βR(CD) attached to 50 μl of 50% (v/v) glutathione-agarose. Finally, the glutathione-agarose beads were washed six times with lysis buffer. The sample was fractionated by SDS-PAGE (10% (v/v) acrylamide). Gels were dried and analyzed by autoradiography.

In Vitro Kinase Assay—In vitro kinase assays were carried out as described by Darnay et al. (21). Following incubation with HepG2 cell lysates GST-LT-βR(CD) was incubated with 50 μl of kinase reaction buffer (20 mM HEPES, pH 7.4, 1 mM MgCl2, 0.2 mM NaF, 0.1 mM sodium orthovanadate) and 10 μCi of [γ-32P]ATP for 10 min at 37 °C, in the presence or absence of Mg2+ or Mn2+. Reactions were stopped by addition of Laemmli’s sample buffer. Samples were fractionated by SDS-PAGE (10% (v/v) acrylamide), and proteins were visualized by Coomassie Blue staining. Phosphorylated proteins were identified by autoradiography.

In-gel Kinase Assay—The in-gel kinase assay was modified from that developed by Kameshita and Fukushima (27). Samples were fractionated on SDS-polyacrylamide gel (10% (v/v) acrylamide) containing GST-LT-βR(CD) fusion protein. The gel was then washed with 20% (v/v) propan-2-ol to remove SDS. The gel was denatured with 6 M guanidine HCl, then renatured in 50 mM Tris-HCl, pH 8.0, 0.04% (v/v) Tween 40. This was followed by washing at 4 °C for 30 min with two changes of buffer (20 mM HEPES, pH 7.4, 0.2 mM NaF, 0.1 mM sodium orthovanadate), and incubation at 22 °C for 3 h in the kinase reaction buffer containing 50 μCi of [γ-32P]ATP. After removal of unreacted [γ-32P]ATP, protein kinases were visualized by autoradiography of the dried gel. Signals due to protein kinases were also detected and quantified using a PhosphoImage™ (Molecular Dynamics) to allow comparison of signal strengths.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was carried out as described by et al. (28). Samples were transferred to Immobilon™-P membrane after fractionation by SDS-PAGE (10% (v/v) acrylamide). A strip of membrane containing phospho-GST-LT-βR(CD) was hydrolyzed in 6× HCl for 90 min at 110 °C, then dried by speed vacuum. After resuspension in H2O, samples were spotted onto Whatman KC, ethyl reverse-phase thin layer chromatography (TLC) plates and then analyzed by ascending TLC in buffer containing 4% (v/v) methanol, 1.5% (v/v) acetic acid, and 0.5% (v/v) formic acid. The migration standards were visualized by ninhydrin staining.

RESULTS

Kinase Activity Associated with GST-LT-βR(CD)—To identify and characterize the proteins associated with LT-βR, we fused the cytoplasmic domain of LT-βR with glutathione-S-transferase and expressed the GST-LT-βR(CD) fusion protein in Escherichia coli. After purification with glutathione-conjugated agarose beads, a protein migrating at 50 kDa was observed by SDS-PAGE. This corresponds to the predicted molecular mass of the fusion protein. Since HepG2 cells have been shown to contain abundant LT-βR receptors, both by reverse transcription-PCR and immunoprecipitation analysis, the purified GST-LT-βR(CD) fusion protein was incubated with [35S]cytochrome/methionine-labeled HepG2 cell lysates to identify proteins that bind to the cytoplasmic domain of LT-βR. After incubation, several proteins with molecular mass values of 75–80, 61, 50, 43, 34, and 29 kDa were found to be associated specifically with GST-LT-βR(CD) (Fig. 1, lane 3), but not with GST under the same conditions (Fig. 1, lane 2) or with GST-LT-βR(CD) in the absence of HepG2 cell lysates (data not shown).

To test for the presence of kinase activity in the proteins associated with GST-LT-βR(CD), we carried out an in vitro kinase assay. As shown in Fig. 2, a 50-kDa protein corresponding to the molecular mass of GST-LT-βR(CD) was phosphorylated after incubation with HepG2 cell lysates in the in vitro kinase assay (Fig. 2B, lane 1). However, there was no evidence that GST was phosphorylated under the same condition (Fig. 2B, lane 3), nor was the GST-LT-βR(CD) fusion protein phosphorylated in the absence of HepG2 cell lysates (Fig. 2B, lane 2). This result suggests that the cytoplasmic domain of LT-βR does not have intrinsic kinase activity, but that it might associate with one or more kinases found in HepG2 cell lysates. The smaller phosphorylated proteins (visible in lane 1 of Fig. 2B) may be phosphoproteins associated with GST-LT-βR(CD), but are more likely to be due to degradation of GST-LT-βR(CD).
since the same species were also observed on the SDS-polyacrylamide gel following Coomassie Blue staining (Fig. 2A). Furthermore, an 80-kDa phosphoprotein (p80) was found to associate with GST-LT-βR(CD) in the in vitro kinase assay (Fig. 2B, lane 1). Similar results were obtained using lysates from other cell lines expressing LT-βR, including K562, U937, and HeLa (data not shown).

**Serine/Threonine Kinase Activity Associated with LT-βR—**

The LT-βR-associated kinase activity was found to be optimal in the presence of Mg\(^{2+}\) or Mn\(^{2+}\) (Fig. 3A) and was inhibited by the serine/threonine protein kinase inhibitor staurosporine. In contrast, the kinase activity was not inhibited by calphostin C, which is highly specific inhibitor of protein kinase C. In addition, neither genistein nor tyrphostin A25 inhibited the LT-βR(CD)-associated kinase activity, although both of these reagents have a broad spectrum inhibitory effect on protein tyrosine kinases (Fig. 3B). Phosphoamino acid analysis showed that serine residues were phosphorylated as were threonine residues, but to a lesser extent (Fig. 3C). However, there was no incorporation of phosphate into tyrosine residues. These observations provide direct evidence that a serine/threonine kinase activity associates with the cytoplasmic domain of LT-βR.

**Characterization of the Substrate Specificity of the LT-βR-associated Kinase—**

It has been shown that the TNFRII-associated kinase can phosphorylate the cytoplasmic domain of TNFRI, but not vice versa (21). We, therefore, investigated whether the LT-βR-associated kinase could phosphorylate TNFRI, which is abundantly expressed in HepG2 cells (30). As shown in Fig. 4, A and B, the kinase associated with the GST-LT-βR(CD) deletion mutant Δ6 (amino acids 324–377, Fig. 6) could phosphorylate the 50-kDa wild type GST-LT-βR(CD) (Fig. 4, A and B, lane 3), while GST and the 60-kDa GST-TNFRI(CD) fusion protein could not be phosphorylated under the same conditions (Fig. 4, A and B, lanes 1 and 2). The kinase activity associated with GST-LT-βR(CD) was able to phosphorylate other substrates, such as histone H1 and myelin basic protein (MBP), but not casein and bovine serum albumin (Fig. 4, C and D).

**Characterization of the LT-βR-associated Kinase by In-gel Kinase Assay—**

To further characterize the kinase activity associated with LT-βR, an in-gel kinase assay was performed. With GST-LT-βR(CD) as substrate, two signals of 50 and 80 kDa (Fig. 5A, lane 4) were observed, corresponding to proteins specifically associated with LT-βR, while a third protein of 82 kDa was also found to associate with both GST (Fig. 5A, lane 3) and GST-LT-βR(CD) (Fig. 5A, lane 4). This result demonstrates the presence of two kinases, p50 and p80, specifically associated with LT-βR. To determine whether these two kinases are able to autophasporlyate, the same experiment was carried out in the absence of substrate in the gel. A similar result was observed, but the signals detected were less than one-tenth of the strength of those seen when GST-LT-βR(CD) was present in the gel (Fig. 5B). Based on the observations above, it is clear that both p50 and p80 are LT-βR(CD)-associated kinases, which are able to autophasporlyate and to mediate phosphorylation of LT-βR. An in-gel kinase assay was also carried out with MBP as substrate, and signals of 50 kDa and 80 kDa were observed (data not shown), confirming that p50 and p80 can phosphorylate MBP.

**Kinase Activity Associated with Amino Acid Residues 324–377 of LT-βR—**

We wished to identify the region(s) of LT-βR to which the novel kinases, p50 and p80, bind. For this purpose, we constructed several deletion mutants of GST-LT-βR(CD) (Fig. 6A) and expressed the resultant fusion proteins in E. coli (Fig. 6B). No phosphoproteins were detected following in vitro kinase assays using the deletion mutants Δ1, Δ8 (Fig. 6C), Δ2, and Δ7 (data not shown). In contrast, it is clear that kinase activity does associate with deletion mutants Δ3, Δ4, Δ5, and Δ6 (Fig. 6C). These results suggest that the minimal region for kinase association is contained within the 54 amino acids present in deletion mutant Δ6. It is possible that other regions of LT-βR might be able to associate with the putative kinase, but are not phosphorylated by it. To rule out this possibility, we

![Fig. 1](image1.png)

**Fig. 1. Proteins associated with GST-LT-βR(CD).** Lysates of [35S]Cys/Met-labeled HepG2 cells (1 × 10^7^) were preincubated with GST bound to glutathione-agarose beads (lane 1) to remove any proteins that associate with GST. The cell lysates were then incubated with GST-adsorbed (lane 2) or GST-LT-βR(CD) adsorbed (lane 3) glutathione-agarose beads. The beads were recovered and, after washing, samples were fractionated by SDS-PAGE (10% (w/v) acrylamide). An autoradiograph of the gel is shown. Proteins specifically associated with GST-LT-βR(CD) and their molecular mass in kDa are indicated on the right of the figure. Molecular mass standards are indicated on the left.

![Fig. 2](image2.png)

**Fig. 2. Kinase activity associated with GST-LT-βR(CD).** GST-LT-βR(CD) (lane 1) or GST (lane 2) incubated with HepG2 cell lysates (2 × 10^7^) or GST-LT-βR(CD) alone (lane 2) were subjected to in vitro kinase assays and then fractionated by SDS-PAGE (10% (w/v) acrylamide). Proteins were detected by Coomassie Blue staining (A) or autoradiography (B).
incubated the deletion mutants D1, D2, D7, and D8 with HepG2 cell lysates. We then performed in vitro kinase assays with wild type GST-LT-βR(CD) as substrate. GST-LT-βR(CD) was not phosphorylated under these conditions (data not shown). Therefore, we conclude that the minimal region for kinase association is located between amino acids 324 and 377 of LT-βR. It is interesting to note that both p50 and p80 were also detected when using deletion mutants D3, D4, D5, and D6 as baits in the in vitro kinase reaction, phosphoamino acid analysis of GST-LT-βR(CD) was carried out by ascending thin layer chromatography. Migration of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) standards is indicated.

incubated the deletion mutants Δ1, Δ2, Δ7, and Δ8 with HepG2 cell lysates. We then performed in vitro kinase assays with wild type GST-LT-βR(CD) as substrate. GST-LT-βR(CD) was not phosphorylated under these conditions (data not shown). Therefore, we conclude that the minimal region for kinase association is located between amino acids 324 and 377 of LT-βR. It is interesting to note that both p50 and p80 were also detected when using deletion mutants Δ3, Δ4, Δ5, and Δ6 as baits in the in vitro kinase assays, but were absent when using Δ1, Δ2, Δ7, and Δ8 in the same experiment (Fig. 6C). This result supports the co-existence of kinase activity with p50 and p80.

**DISCUSSION**

Using the yeast two-hybrid and GST fusion protein systems, many proteins have been shown to associate with TNFRs. These include: the tumor necrosis receptor-associated factors, TRAF1–6 (16, 17, 31–33); tumor necrosis factor receptor-associated proteins, TRAP-1 and TRAP-2 (34); proteins containing death domains, such as TRADD and RIP (25, 35); p60-TRAK, a TNFRI-associated kinase (21); and p80-TRAK, a TNFRII-associated kinase (22). Among these TNFR-associated proteins, however, only TRAF3 and TRAF5 have been shown to associate with LT-βR (16, 17). Therefore, we wished to determine whether there are other proteins associated with LT-βR. Using GST-LT-βR(CD) as a bait, we have identified several proteins with molecular mass values of 75–80, 61, 50, 43, 34, and 29 kDa that associate with GST-LT-βR(CD) (Fig. 1). Based on molecular mass, the 61-kDa protein might correspond to TRAF3 (62 kDa) and/or TRAF5 (64 kDa). In addition, the results of both in-gel kinase assays and in vitro kinase assays show the presence of two kinases, p80 and p50, associated with the cytoplasmic domain of LT-βR (Figs. 5 and 6). It is likely that the 80- and 50-kDa proteins from [35S]cysteine/methionine-labeled HepG2 cell lysates, that bound to GST-LT-βR(CD) (Fig. 1), correspond to the two LT-βR-associated kinases, p80 and p50, respectively.

We are confident that the LT-βR-associated kinases are distinct from the serine/threonine kinases that associate with TNFRI and TNFRII for the following reasons. (i) The LT-βR-associated kinases are distinct from the serine/threonine kinases that associate with TNFRI and TNFRII for the following reasons.
associated kinases could only use LT-βR and not TNFRI as substrate in the in vitro kinase assay; (ii) the TNFRI-associated kinase is known to phosphorylate TNFRI, but this phenomenon was not observed for the LT-βR-associated kinases; (iii) the activity of the LT-βR-associated kinases could be inhibited by staurosporine, while the TNFRI-associated kinase activity is only inhibited by CKI-7 and not by staurosporine (26); (iv) the specificities of these receptor-associated kinases for other standard kinase substrates, such as histone H1, casein, and MBP, are all distinct from each other. The LT-βR-associated kinases can phosphorylate histone H1 and MBP, but not casein. By contrast, the TNFRI-associated kinase can phosphorylate both casein and histone H1, but not MBP (21), while the TNFRI-associated kinase can use MBP as substrate, but not casein or histone H1 (22).

Receptor clustering is crucial in the activation of the TNFRs. Using the yeast two-hybrid system, TNFRI (but not TNFRII) has been shown to self-associate (36, 37), and it has been suggested that this is a consequence of receptor phosphorylation. Darnay et al. (24) used GST-TNFRI as bait to identify two phosphoproteins, p55 and p58, associated with TNFRI. The molecular masses of these species suggested that they may correspond to endogenous TNFRI (60 kDa). However, this could not be proved because the anti-TNFRI antibody fails to detect either p55 or p58 on an immunoblot (24). Using a similar approach, we found an 80-kDa phosphoprotein associated with GST-LT-βR(CD) in the in vitro kinase assay. Since the self-association of the intracellular domain of LT-βR has been observed in the yeast two hybrid system, it was possible that p58 corresponded to the endogenous LT-βR, which has a molecular mass of 75–80 kDa (38). To test this, we used anti-LT-βR antiserum to pre-clear endogenous LT-βR from HepG2 cell lysates before incubation with GST-LT-βR(CD). In subsequent in vitro kinase assays, the intensity of the p58 phosphoprotein was unchanged despite the complete removal of endogenous LT-βR from the HepG2 cells (data not shown). Therefore, we conclude that the p58 phosphoprotein is not the endogenous LT-βR.

The kinase activities associated with TNFRI and TNFRII have been shown to be up-regulated following TNF stimulation (21–23). Therefore, the effect of ligand-receptor interaction on the LT-βR-associated kinase activity was examined by treating HepG2 cells with either immobilized anti-LT-βR antibodies or activated RAMOS cells. The latter have been shown to express the membrane-bound LTα1/β2 heterotrimer following phorbol 12-myristate 13-acetate stimulation (6). However, the LT-βR-associated kinase activities were not affected by stimulation with either anti-LT-βR or phorbol 12-myristate 13-acetate-activated RAMOS cells (data not shown).

When using an anti-LT-βR monoclonal antibody to precipitate endogenous LT-βR from HepG2 cells for the in vitro kinase assay, we observed that the endogenous LT-βR could only be phosphorylated after treatment of the immunoprecipitates with alkaline phosphatase. This suggests that LT-βR is constitutively phosphorylated in the intact cells and supports the argument that the kinase activity could be co-precipitated with endogenous LT-βR, which is the primary target for its associ-

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3 Y. H. Wu-Lee, personal communication.
4 M.-Y. Wu and S.-L. Hsieh, unpublished data.
phosphorylation of this receptor following TNF stimulation. To further understand the roles of these
remains to be elucidated.

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