NF-κB-inducing kinase (NIK) has been shown to play an essential role in the NF-κB activation cascade elicited by lymphotoxin β receptor (LTβR) signaling. However, the molecular mechanism of this pathway remains unclear. In this report we demonstrate that both NIK and Ixk kinase α (IKKα) are involved in LTβR signaling and that the phosphorylation of the p65 subunit at serine 536 in its transactivation domain 1 (TA1) plays an essential role. We also found that NF-κB could be activated in the LTβR pathway without altering the level of the phosphorylation of IxkB and nuclear localization of p65. By using a heterologous transactivation system in which Gal4-dependent reporter gene is activated by the Gal4 DNA-binding domain in fusion with various portions of p65, we found that TA1 serves as a direct target in the NIK-IKKβ pathway. In addition, mutation studies have revealed the essential role of Ser-536 within TA1 of p65 in transcriptional control mediated by NIK-IKKα. Furthermore, we found that Ser-536 was phosphorylated following the stimulation of LTβR, and this phosphorylation was inhibited by the kinase-dead dominant-negative mutant of either NIK or IKKα. These observations provide evidence for a crucial role of the NIK-IKKα cascade for NF-κB activation in LTβR signaling.

The lymphotoxin (LT) signals crucial roles in the embryonic development of lymphoid organ, the maintenance of lymphoid architecture, and the formation of ectopic lymphoid tissue adjacent to chronic inflammatory sites (1–3). LT is a heterotrimer complex consisting of α (LTα) and β (LTβ) subunits (as LTα2β2), which bind to its specific receptor (LTβR) (1). LTβR signaling involves NF-κB-inducing kinase (NIK), which eventually activates nuclear factor κB (NF-κB) (4, 5). The involvement of NIK in the LTβR signaling has been suggested by the shared phenotypes of gene knock-out mice of LTα, LTβ, and LTβR (1, 6, 7) and lymphoplasia mice (aly/aly) (8) in which spontaneous mutations of NIK were found responsible (9–11). Moreover, LTβR signaling was shown to involve both NIK and IKKα for NF-κB activation (4). It has also been shown that NIK is indispensable for LTβR signaling but not for tumor necrosis factor (TNF) signaling (5). However, the molecular mechanism by which NF-κB is activated by LTβR signaling has not been clarified.

NF-κB represents a family of eukaryotic transcription factors participating in the regulation of immune response, cell growth, and survival (12–16). There are five members of the NF-κB/Rel family in mammalian cells: the proto-oncogene c-Rel, RelA (p65), RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). The most prevalent form of NF-κB is a heterodimer of the p50 subunit and p65 that contains transactivation domains necessary for gene induction (17–20).

In cells, NF-κB is largely cytoplasmic and therefore remains transcriptionally inactive until a cell receives an appropriate stimulus. In response to proinflammatory cytokines such as TNF and interleukin-1β (IL-1β), the IxkB proteins become phosphorylated on two serine residues located in the N-terminal region (21). Phosphorylation of IxkB proteins results in rapid ubiquitination and subsequent proteolysis by the 26 S proteasome (15, 22, 23), which allows the liberated NF-κB to translocate to the nucleus and participate in target gene transcriptional regulation. In cells, NF-κB is largely cytoplasmic and therefore remains transcriptionally inactive until a cell receives an appropriate stimulus. In response to proinflammatory cytokines such as TNF and interleukin-1β (IL-1β), the IxkB proteins become phosphorylated on two serine residues located in the N-terminal region (21). Phosphorylation of IxkB proteins results in rapid ubiquitination and subsequent proteolysis by the 26 S proteasome (15, 22, 23), which allows the liberated NF-κB to translocate to the nucleus and participate in target gene transcriptional regulation (12–15). The large molecular weight complex consisting of two catalytic subunits, IxkB kinases α and β (IKKα and IKKβ), and a regulatory subunit IKKγ was identified and shown to be responsible for phosphorylating IxkB proteins (24–29). It has recently been shown that IKKα is not required for IxkB degradation or induction of NF-κB DNA binding but essential for the generation of transcriptionally competent NF-κB (30). The kinase activity of IKKs is induced by a wide variety of NF-κB inducers such as TNF or IL-1β, and mediated by the upstream kinases including NIK and the extracellular signal-regulated kinase kinase kinase 1/3 (31–34). NIK was originally identified as a protein interacting with the TNF receptor-associated factor 2 component of the TNF receptor complex (35). NIK physically interacts via its C-terminal region with IKKα and IKKβ and stimulates their catalytic activity as an upstream effector kinase (32, 36–39).

The NF-κB p65 subunit contains at least two independent transactivation (TA) domains (TA1 and TA2) within its C-terminal 120 amino acids and is responsible for binding to the basal transcription factor TFIIa and CBP/p300 coactivators (19, 20). The TNF-mediated signaling was shown to involve phosphorylation of Ser-529 within TA1 by casein kinase II (CKII) (40, 41). Similarly, overexpression of IKKβ induced phosphorylation of p65 at Ser-536 (42). These two serine residues within p65 TA1 were also shown to be essential for Ras-mediated NF-κB activation involving phosphatidylinositol 3-ki-
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**Experimental Procedures**

*Plasmid Constructs—* Mammalian expression vectors, pM-p65, pM-p65 (286–521), pM-p65 (286–520), pM-p65 (265–521), and pDNA3.1-p65 were created as previously described (38). pM-p65 (1–268) was generated by amplifying the corresponding p65 fragment by PCR using the oligonucleotide primers 5′-CCGAGATCCGCCAGGCTCAGAGAAGATGCGATGACGACAAGATGGACGAACTGTTCCCCCTCAT-3′ and 5′-GCTTCTCGAGTACCAAAATGCATGTCCGCAATGGC-3′ containing four tandem copies of the HIV TATA box was used for the evaluation of the transcriptional activity of luc genes. All the plasmids used in this work were created using the appropriate oligonucleotides and PCR (New England Biolabs). All the constructs were confirmed by dideoxynucleotide sequencing using ABI PRISM™ dye terminator cycle sequencing ready kit (PerkinElmer Life Sciences) on an Applied Biosystems 3130 automated DNA sequencer.

**Western Blotting**—To detect the phosphorylated p65 at Ser-536,mediated by NIK-IKKα, is crucial for LTβR signaling.

**Immunostaining**—The intracellular localization of p65 in HT29 cells was examined by immunostaining as described previously (50). Briefly, HT29 cells were cultured in 2-well chamber slides and after stimulating with 10 ng/ml of TNF for 15 min or 2 μg/ml of agonistic anti-LTβR mAb for 40 min, cells were fixed in 4% (w/v) paraformaldehyde/PBS at room temperature for 20 min and then permeabilized with 0.5% Triton X-100/ PBS for 20 min at room temperature. They were then incubated with rabbit polyclonal antibody against p65 (Santa Cruz Biotechnology) for 1 h at 37 °C, rinsed three times with 0.05% Triton X-100/PBS, and incubated with secondary antibody, fluorescein-conjugated goat anti-rabbit IgG (CAPPEL; ICN Pharmaceuticals), for 1 h at 37 °C. The slides were rinsed three times with PBS and mounted with buffered glycerol for fluorescent microscopic examination. Primary and secondary antibodies were diluted at 1:100 and 1:200 in PBS containing 3% bovine serum albumin, respectively.

**RESULTS**

**Activation of NF-κB-dependent Gene Expression by TNF and LTβR Signaling**—In Fig. 1A, the effects of TNF and LTβR signaling on NF-κB-mediated gene expression were compared. TNF stimulated NF-κB-dependent gene expression in both 293 and HT29 cells. However, the agonistic LTβR mAb stimulated gene expression only in the LTβR-expressing HT29 cells as reported (54). Overexpression of NIK stimulated gene expression in both cells, indicating that the differences in these cells depend on...
LTβR. In addition, overexpression of IKKα alone did not significantly activate the NF-κB-dependent gene expression in both cells whereas that of IKKβ activated the gene expression by 3- and 1.8-fold in 293 and HT29 cells, respectively. In fact, whereas IKKβ overexpression induced IκBα degradation, IKKα overexpression did not (data not shown). These findings suggested that the upstream signal is required for optimal activation as previously reported (24, 26).

As demonstrated in Fig. 1B, TNF stimulation induced the phosphorylation of IκBα and subsequent degradation in HT29 cells. However, stimulation of LTβR did not induce either IκBα phosphorylation or its degradation, yet induced NF-κB-dependent gene expression. Moreover, whereas TNF induced the nuclear translocation of NF-κB in HT29 cells, LTβR did not (Fig. 1C). The presence of NF-κB in the nucleus even in the resting cells has been demonstrated recently by Birbach et al. (44) (see also Ref. 55 for review). These findings suggest that the LTβR signaling stimulates NF-κB activity without inducing IκBα degradation or NF-κB nuclear translocation as reported by Yin et al. (5) and that the activation of NF-κB by the LTβR signaling is not through alteration of intracellular localization of NF-κB but presumably by augmenting its transcriptional activity.

NIK-IKKα Plays a Key Role in Regulating the Transcriptional Activity of NF-κB during LTβR Signaling—In a series of experiments using transient κB luciferase assays, we have explored the kinase responsible for NF-κB activation in the LTβR cascade by using the dominant-negative kinase mutants of NIK (NIK(KM)), IKKα (IKKα(KM)), and IKKβ (IKKβ(KM)). As demonstrated in Fig. 2A, when NIK(KM), IKKα(KM), or IKKβ(KM) were overexpressed in 293 cells, the LTβR-induced NF-κB activation was greatly inhibited by either of these mutants, more remarkable by IKKβ(KM). A similar observation was obtained with HT29 cells (data not shown). These results are consistent with the fact that the TNF-mediated NF-κB activation was abolished in the IKKβ gene knock-out mice but could not entirely be abolished in IKKα and NIK knock-out mice (5, 56–60). Interestingly, the induction of NF-κB-dependent gene expression by agonist anti-LTβR mAb was strongly inhibited by NIK(KM) or IKKα(KM) in HT29 cells but not by IKKβ(KM). In Fig. 2B, synergistic activation of gene expression was investigated. When wild-type NIK, IKKα, or IKKβ were overexpressed together with TNF signaling in 293 cells, there was no significant augmentation by IKKα as compared from TNF alone. However, either NIK or IKKβ augmented the effect of TNF in inducing NF-κB-dependent gene expression, which were statistically significant (p < 0.01 and p < 0.05, respectively). On the other hand, in HT29 cells, the gene expression elicited by anti-LTβR mAb was augmented significantly by IKKα and NIK (p < 0.05 and p < 0.01, respectively) but not at
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**Fig. 2.** The essential roles of NIK and IKKα in the transcriptional activation of NF-κB induced by LTβR signaling. A, distinct inhibition profiles by kinase-defective mutants of NIK and IKK in the LTβR and TNF signaling. Their effects on the NF-κB-dependent luc gene expression were evaluated. Expression plasmids for dominant-negative NIK (pcDNA3-NIK(KM)), IKKα (pCR2FL-IKKα(KM)), or IKKβ (pCR2FL-IKKβ(KM)) were cotransfected with 4× xBw-luc, and the extent of stimulation was compared when NF-κB was activated either by TNF or agonistic anti-LTβR mAb. After transfection with the indicated plasmids, 293 cells and HT29 cells were stimulated by TNF (10 ng/ml) for 24 h and anti-LTβR mAb (2 μg/ml) for 10 h, respectively. Note that the LTβR signaling was blocked by NIK(KM) or IKKα(KM) but not by IKKβ(KM). B, synergy between the signaling effectors in NF-κB activation. In 293 cells, the synergistic activation was examined between TNF and wild-type NIK, IKKα, or IKKβ. Similarly, in HT29 cells, the synergistic activation was examined between anti-LTβR mAb and IKKα or IKKβ. C, synergy between NIK and the downstream kinases in the NF-κB activation. NIK was overexpressed together with wild-type IKKα, IKKβ, or their kinase-defective mutants, and the effect on NF-κB-dependent gene expression was determined. Note that IKKα and IKKβ augmented the effect of NIK, yet only IKKα(KM) inhibited the effect of NIK. There was no effect of IKKα, IKKβ, or NIK overexpression on the levels of endogenous p65 (data not shown). The data are presented as the fold increase in luciferase activities (mean ± S.D.) relative to the control of three independent transfections.

All by IKKβ. These data collectively indicated that the TNF-induced NF-κB activation is mainly through IKKβ but the NF-κB activation in LTβR pathway is mediated by NIK and IKKα, which was consistent with the previous study (4, 5).

The results of Fig. 2C demonstrated that the synergy between NIK and IKKα or IKKβ was observed irrespective of the presence or absence of LTβR in cells. Moreover, the abolishment of the effect of NIK by IKKα(KM), not by IKKβ(KM), was observed equally in both cells. These observations suggest that activation of NIK is mainly coupled with IKKα but not IKKβ.

Involvement of the p65 C-terminal TA Domain in Signaling Mediated by NIK-IKKα—Since NIK-IKKα was shown to activate the NF-κB-dependent gene expression by augmenting the transcriptional activity of NF-κB independently of the IκB degradation pathway, we examined whether the p65 subunit is directly involved. In Fig. 3, we adopted a heterologous luciferase reporter system with Ga4-luc from which gene expression is under the control of Gal4. As shown in Fig. 3B, pm-p65, expressing the Gal4-p65 (full-length) fusion protein, augmented the gene expression from the Gal4-dependent promoter when NIK was overexpressed.

In order to determine which portion of p65 is responsible for this action of NIK, we have created a series of plasmids expressing various portions of p65 in fusion with the Gal4 DNA-binding domain (Fig. 3A): pm-p65, containing full-size p65(1–551); pm-p65(1–286), containing RHD; pm-p65(286–551), containing NLS but lacking RHD; pm-p65(286–551), containing RHD but lacking NLS and TA2; pm-p65(286–520), containing NLS but lacking RHD and TA1; pm-p65(431–551), containing only TA2 and TA1; pm-p65(521–551), containing only the TA1 domain of p65. In Fig. 3B, NIK stimulated the p65 (full-length)-mediated transactivation by 5.8-fold. Likewise, NIK stimulated the effect of pm-p65(286–551), pm-p65(286–551), pm-p65(286–551), and pm-p65(286–520), by 3.2-, 3.2-, and 3.4-fold, respectively. Interestingly, NIK stimulated pm-p65(431–551), containing only TA2 and TA1, by 6.3-fold, and its transcriptional activity as well as the susceptibility to the NIK-mediated activation was similar to pm-p65 (containing full-size p65). Moreover, the Gal4-p65(521–551) containing
only the TA1 domain had the highest susceptibility for the NIK-mediated transactivation (19.7-fold, comparing lanes 15 and 16) although the basal transcription level was relatively low. pM-p65-(1–286) containing only RHD and pM-p65-(286–430) lacking both TA2 and TA1 supported no effect of NIK. These results suggested that the effect of NIK was mainly mediated by TA1.

As it was demonstrated that the activation of NIK was coupled with IKKα (4, 61) (Fig. 2C), we next examined whether the dominant-negative IKKα mutant could block these effects of NIK. As shown in Fig. 3C, NIK-mediated activation of the transcriptional activity of Gal4-p65 fusion proteins was inhibited by the overexpression of IKKα(KM). There was no significant effect with IKKβ(KM) (data not shown). Similar results were obtained with HT29 cells (data not shown). These data collectively demonstrated that NF-κB transcriptional activation elicited by NIK-IKKα was mediated through the C-terminal TA1 domain of p65.

Serine 536 in the p65 TA1 Domain Is Responsible for the Effect of NIK—Since the effect of NIK-IKKα on p65 was primarily mediated by the TA1 domain of p65, we further examined the effect of mutation in Ser-536 within TA1. We also addressed whether IκBa could block the effect of NIK since it was recently demonstrated that IκBa is present in the nucleus and exhibits the inhibition of NF-κB transcriptional activity (41, 42, 44–47). In Fig. 4, the Gal4-luc reporter plasmid was co-transfected with pM-p65-(521–551), pM-p65-(521–551:S529A), and pM-p65-(521–551:S536A) with or without pcDNA3-NIK (expressing the wild-type NIK). When IκBa2AN (a superactive mutant of IκBa) was expressed, the effect of NIK was inhibited. Although NIK stimulated the transcriptional activities of pM-p65-(521–551) and pM-p65-(521–551:S529A) similarly as in Fig. 3, B and C, the extent of stimulation was significantly reduced with pM-p65-(521–551:S536A), indicating that Ser-536 is indispensable for the transcriptional activity of p65 in response to the LTβR signaling mediated by NIK-IKKα. These findings indicated that the effect of NIK on the p65 TA1 domain might depend on the phosphorylation of p65 at serine 536, and this action of NIK could be inhibited by IκBa.

Phosphorylation of p65 at Ser-536 in LTβR Pathway—In Fig. 5, we further examined whether Ser-536 in p65 is essential for the LTβR signaling involving NIK and IKKα, and could be phosphorylated in HT29 cells when stimulated with the agonist anti-LTβR mAb. We first addressed whether the mutant p65, in which Ser-536 is substituted by Ala, is still responsive to LTβR signaling. As demonstrated in Fig. 5A, although anti-LTβR mAb stimulated the transcriptional activity of pM-p65-(521–551), its action was completely abolished when Ser-536 was substituted by Ala. In addition, when dominant-negative mutants of NIK and IKKα were expressed, this action of LTβR signaling was blocked, suggesting that the effect of LTβR is mediated by NIK and IKKα leading to the phosphorylation at Ser-536 in p65. We examined more directly whether Ser-536 is phosphorylated in response to the LTβR signaling (Fig. 5B). When full-length p65 (FLAG-tagged) was expressed, Ser-536 phosphorylation was detected by the specific antibody (anti-phospho-p65 NF-κB (Ser-536)), and this phosphorylation was blocked upon coexpression of dominant-negative mutants

IKKα(KM) inhibited the effect of NIK on the transcriptional activity of Gal4 fusion p65 mutants. 293 cells were transfected with Gal4-luc, pcDNA3-NIK in the presence or absence of pCR2FL-IKKα(KM) (50 ng) together with various pM-p65 constructs. The data are presented as the relative fold changes relative to control transfection of three independent experiments. Similar observation was obtained in HT29 cells (data not shown).
of NIK or IKKα, or phosphodefective IκBα (Myc-tagged IκBα(S32A/S36A)). These data clearly indicate that LTβR signaling eventually leads to the phosphorylation of p65 at Ser-536. Both NIK and IKKα are involved, and this process can be blocked by IκBα.

**DISCUSSION**

In this study we have explored a mechanism by which NF-κB is activated by LTβR signaling. We found that both NIK and IKKα are critically involved in this pathway since either the kinase-deficient mutant of NIK or IKKα, but not IKKβ, could block LTβR-mediated NF-κB activation and that the LTβR-mediated NF-κB activation did not induce phosphorylation and subsequent degradation of IκBα. We also found that serine 536 phosphorylation in its TA1 transactivation domain is essential. These findings collectively demonstrate the presence of a novel signaling mechanism in the NF-κB activation, which is unique to the LTβR signaling.

Matsushima et al. (4) found that NIK and IKKα were indispensable for NF-κB activation in the LTβR signaling using aly/aly mice in which formation of the secondary lymphoid tissues was affected although the TNF- and IL-1-mediated NF-κB activation signaling remained intact. Similar observations were reported with IKKα-deficient mice (57) and NIK-deficient mice (5). In addition, Cao et al. (62) has recently reported another interesting feature of the IKKα pathway with the mutant IKKα knock-in mice that the signal transduction of receptor activator of NF-κB (RANK) leading to NF-κB activation was abolished and thus the inducible expression of target cyclo D1 gene was affected. Whereas the responsiveness to proinflammatory stimuli including TNF, IL-1, and LPS are largely dependent on the IKKβ, other stimuli such as LTβ, RANK ligand, and Blys/BAFP depend on IKKα-mediated signaling (55). These biological features of IKKα explain the characteristic developmental defect of the secondary lymphoid tissues in the IKKα knock-out mice. Thus, although IKKα and IKKβ are cross-related and both serve as the catalytic subunits of IKK complex, these findings illustrate the functional heterogeneity of IKKα and IKKβ.

Although a major step that regulates NF-κB activity is the removal of IκB from the NF-κB/IκB complex, the capacity of nuclear NF-κB to drive transcription is also a regulated process. A number of studies support the possibility that p65 phosphorylation regulates the transcriptional competence of nuclear NF-κB (41, 63–67). Although the role of PKA in phosphorylating p65 is still controversial (68–70), regulation of the transcriptional competence of p65 by phosphorylation has been widely accepted. Protein kinases such as CKII, PKCζ, and IKK have been implicated in this process (40–42, 67). For example, Wang and Baldwin (40) reported that the phosphorylation of Ser-529 at the TA1 domain of p65 is associated with the TNF-induced NF-κB activation. They later found that CKII interacts with p65 and directly phosphorylates p65 at Ser-529 (41). In addition, Sakurai et al. (42) reported that TNF induced phosphorylation of p65 at Ser-536 in the cell and showed that the p65 could be phosphorylated at Ser-536 by IKKβ at least in vitro. Moreover, Madrid et al. (43) have recently demonstrated that phosphatidylinositol 3-kinase activates Akt, which subsequently activates IKKα and leads to p65 phosphorylation at Ser-536.

One of the possible mechanisms of p65 phosphorylation at its TA domain in controlling its transcriptional competency is to recruit coactivator proteins such as histone acetyl transferases (71, 72) and TLS (73) to NF-κB when it binds to the target promoter sequence. Alternatively, p65 phosphorylation may preclude the recruitment of corepressor proteins such as Groucho family proteins that is known to interact with the p65 TA domain (48) and histone deacetylases (HDACs) (74–77). For example, it was reported that cAMP-dependent kinase (PKA)-mediated phosphorylation of p65 caused the p65 association with CBP in vitro (72). The same group has recently demonstrated...
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A Ser-536 mutant of p65 TA1, which could not mediate the effect of LTβR signaling. Together with our findings, it is likely that the p65 subunit of NF-κB is phosphorylated by the NIK/IKKα cascade in the nucleus.

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Stressed with cultured cells that p65 was associated with HDAC1 in unstimulated cells, and it was dissociated from HDAC1 but associated with CBP upon cotransfection with the PKA catalytic subunit (77). Thus, it is possible that p65 phosphorylation may act as a determinant for selecting the interacting partner of NF-κB.

The results in this study revealed that LTβR signaling induced the p65 phosphorylation at Ser-536 by using phosphorylation-specific antibody. This finding was confirmed with the Ser-536 mutant of p65 TA1, which could not mediate the effect of LTβR signaling. Then, where is NF-κB (p65) phosphorylated in the cell? In fact, a number of studies have revealed that NF-κB and IκB shuttle in and out of the nucleus (44–47). Therefore, NF-κB is present in the nucleus even in the unstimulated cells, although to a lesser amount than that in the cytoplasm. More importantly, Birbach et al. (44) found that the treatment of cells with leptomycin B, an inhibitor of CRM1 and a blocking agent of nuclear export, resulted in the nuclear accumulation of NIK and IκKα, but not IκKδ, indicating that these kinases also shuttle between the cytoplasm and the nucleus. IκKα has been initially identified as NIK-interacting protein in yeast two-hybrid screens (36). Thus, IκKα appears to potentially associate with NIK, where the large IκK complex is not found, such as in the nucleus. Interestingly, when IκKα was mutated at lysine 44, the shuttle of IκKα between cytoplasm and nucleus was prevented because it is known that the lysine residue at position 44 was also essential for the kinase activity (44), which is consistent with our observation that either dominant-negative IκKα (IκKαKM) or phosphorylation-defective mutant IκBα (IκBαN) efficiently blocked LTβR signaling. Together with our findings, it is likely that the p65 subunit of NF-κB is phosphorylated by the NIK/IκKα cascade in the nucleus.
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