TWEAK Induces NF-κB2 p100 Processing and Long Lasting NF-κB Activation*

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Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily that has been shown to induce angiogenesis, apoptosis in tumor cells, and NF-κB activation through binding to its receptor, fibroblast growth factor-inducible 14. We have identified TWEAK as an inducer of constitutive NF-κB activation by expression cloning, and we report here sequential regulation by TWEAK of two separate signaling cascades for NF-κB activation, the NF-κB essential modulator-dependent and -independent signaling pathways. Upon TWEAK stimulation, IκBα is rapidly phosphorylated, generating NF-κB DNA-binding complexes containing p100 and RelA in a manner dependent on the canonical IκB kinase complex. Unlike TNF-α, TWEAK stimulation results in prolonged NF-κB activation with a transition of the DNA-binding NF-κB components from RelA- to RelB-containing complexes by 8 h, and the latter remained active in binding at least until 24 h post-stimulation. This long-lasting activation is accompanied by the proteasome-mediated processing of NF-κB2/p100, which does not depend on the NF-κB essential modulator but requires IκB kinase 1 and functional NF-κB-inducing kinase activity. Finally, we show that fibroblast growth factor-inducible 14 with a mutation at its TNF receptor-associated factor (TRAF)-binding site cannot activate NF-κB and that TWEAK fails to induce the p100 processing and IκBα phosphorylation in cells deficient for TRAF2 and TRAF5. Our results thus identify TWEAK as a novel physiological regulator of the non-canonical pathway for NF-κB activation.

The nuclear factor κB (NF-κB) family of transcription factors regulates expression of various genes that participate in the immune, inflammatory, apoptotic, or oncogenic processes (1–7). Mammalian cells express five NF-κB family members: RelA, RelB, c-Rel, NF-κB2/p100/p52, and NF-κB1/p105/p50 (1–5). In resting cells, NF-κB proteins are associated with an IκB protein and retained in the cytoplasm. Upon stimulation, signals are believed to converge on the IκB kinase (IKK) complex, which is composed of two catalytic subunits, IKK1/α and IKK2/β, and a regulatory subunit NF-κB essential modulator (NEMO)/IKKγ (1–5). Activation of the IKK complex leads to phosphorylation of IκB family proteins, resulting in nuclear translocation of NF-κB hetero/homodimers. Activation of the IKK complex is an essential step for NF-κB activation in response to various cytokines including TNF family members, interleukin-1β (IL-1β), and lymphotoxin-β receptor (LTβR) activated the non-canonical NF-κB signaling pathway, which did not require NEMO for NF-κB activation (10–18). In this pathway, IKK1 plays an essential role in the p100 processing (13, 15, 17–19) and subsequent nuclear translocation of RelB-containing NF-κB dimers (10, 12–18). RelB differs in its regulation from the other two NF-κB activators, RelA and c-Rel, that are primarily controlled by small IκB proteins. Previous studies (20) revealed that p100 is associated with RelB in the cytoplasm and inhibits its nuclear localization, but IκBα, IκBβ, or IκBε are not associated with RelB. NF-κB-inducing kinase (NIK) was initially identified as a TNF receptor-associated factor (TRAF) 2-interacting molecule that activated NF-κB (21) and has turned out to be an important mediator for the p100 processing as revealed by use of cells with the aly mutation in the NIK gene that disrupts the interaction of NIK with IKK1 (22). Thus, it is considered that IKK1 and NIK play essential roles in the non-canonical NF-κB signaling pathway.

The TNF superfamily of ligands plays important roles in regulating host defense, inflammation, cell death, autoimmunity, and organogenesis (1–6). TWEAK, a member of this family, is known to induce cell death in some tumor cell lines (23–25), proliferation of human endothelial cells in vitro, and angiogenesis in vivo (26–28). We reported that TWEAK was expressed on interferon-γ-stimulated human monocytes and involved in their cytotoxicity (24). An expression cloning identified Fn14 (29) as the receptor for TWEAK (27). We demonstrated previously that TWEAK up-regulated the expression of IL-8 and monocyte chemotactic protein-1 in human endothelial cells through NF-κB activation, which were suppressed by blocking antibody against Fn14 (28). However, the mechanism by which TWEAK induces NF-κB activation remains largely unknown.

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The abbreviations used are: NF-κB, nuclear factor-κB; BAFF, B cell activating factor; EMISA, electrophoretic mobility shift assay; Fn14, fibroblast growth factor-inducible 14; IκB, inhibitor of NF-κB; IKK, IκB kinase; IL-1β, interleukin-1β; LTβR, lymphotoxin β-receptor; NEMO, NF-κB essential modulator; NIK, NF-κB-inducing kinase; PE, phycocyanin; TNF-α, tumor necrosis factor-α; TRAF, TNF receptor-associated factor; TWEAK, TNF-like weak inducer of apoptosis; MEF2, mouse embryonic fibroblasts; IRES, internal ribosome entry site.

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The cytoplasmic domain of Fn14, like other members of the TNF receptor superfamily, does not contain consensus amino acid sequences characteristic of domains with enzymatic activity (27, 29). Thus, it is likely that proteins interacting with Fn14 mediate NF-κB activation. The TRAF family proteins were identified as signal transducers that bound to several members of the TNF receptor superfamily, leading to the activation of NF-κB and mitogen-activated protein kinases (30). TRAF2, TRAF5, and TRAF6 were shown to activate NF-κB and to be involved in NF-κB activation mediated by these receptors (30). Previous reports (27, 31) showed that the cytoplasmic domain of Fn14 bound to TRAF1, TRAF2, TRAF3, and TRAF5. However, functional significance of these TRAF proteins in Fn14-mediated NF-κB activation is not well established.

We initially isolated TWEAK as an inducer of constitutive NF-κB activation through expression cloning. Subsequent studies have shown that TWEAK stimulation induces rapid and long-lasting NF-κB activation via IκBα and p100 regulation. We further demonstrate how NF-κB regulators contribute to TWEAK-induced NF-κB activation, using IKK1−/−, IKK2−/−, NIK<sup>−/−</sup>, TRAF2<sup>−/−</sup>/− mouse embryonic fibroblasts (MEFs) and NEMO-deficient rat fibroblasts.

**EXPERIMENTAL PROCEDURES**

Reagents—Anti-Fn14 antibody (ITEM-2) and anti-TWEAK antibody (CARL-1) were described previously (24–25, 28, 32). Anti-p-IκBα (Ser32) antibody was purchased from Cell Signaling. Anti-actin antibody (C-2) and anti-p52 antibody (K-27) used for Western blot analysis were purchased from Santa Cruz Biotechnology. Anti-p52 serum (06-413) (C-2) and anti-p52 antibody (K-27) used for supershift assay was purchased from Upstate Biotechnology, Inc. Anti-p50, anti-ReLA, and anti-ReLB sera were kindly provided by Drs. Nancy Rice and Alain Israël (Institut Pasteur, Paris, France). Recombinant CD8-TWEAK fusion protein was prepared as described previously (24). All the reagents were purchased from Sigma unless otherwise noted.

**Cell Culture, Transfection, and Retrovirus**—Rat-1, -5R, and -B5, which is a subline of Rat-1 and expresses the blasticidin deaminase gene under the control of an NF-κB-dependent promoter, were described previously (8, 14, 33). Wild-type and TRAF2<sup>−/−</sup>/− MEFs were described previously (14, 34). IKK1<sup>−/−</sup> MEFs were kindly provided by Drs. Véronique Baud and Michael Karin (University of California, San Diego). IKK2<sup>−/−</sup> MEFs were kindly provided by Dr. Manolis Pasparakis (EMBL, Heidelberg). NIK<sup>−/−</sup> MEFs were kindly provided by Dr. Mitsu Matsumoto (Tokushima University). PLAT-E packaging cells were described previously (35). The cells used in this study were all maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. MEFs and PLAT-E cells were transfected using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. For production of retroviruses, culture supernatants of PLAT-E cells were collected 60 h after transfection with retroviral vectors. B5 and MEFs were infected in the presence of 10 μg/ml of Polybrene.

**Construction of cDNA Library and Screening of NF-κB Regulator—** A Scal/BglII DNA fragment containing the cytomegalo virus early enhancer-promoter, U3-deleted murine leukemia virus-long terminal repeat and packaging signal, was excised from pRhCD255N (36) and subcloned into the same enzymatic site of pMX (37), generating pMRX. Construction of retroviral cDNA library was performed as described previously (38). Briefly, complementary DNAs were synthesized from poly(A)<sup>+</sup> RNA of Rat-1 cells with oligo(dT) primer using SuperScript Choice System (Invitrogen) according to the manufacturer’s instructions. The synthesized cDNAs were inserted between the BstXI sites of pMRX using BstXI adaptors (Invitrogen), generating a retroviral cDNA expression library. B5 cells were infected with retroviruses expressing this cDNA library and then subjected to lethal selection with blasticidin S (5 μg/ml) 36 h after infection. After the selection, genomic DNAs were isolated from surviving cell clones that showed elevated NF-κB activity. Four insert cDNAs were amplified by PCR from the genomic DNA, cloned into pDrive (Qiagen), and sequenced. The TWEAK cDNA was subcloned into pMRX, generating pMRX-TWEAK.

**Construction of Expression Vectors—** A NotI/SalI DNA fragment containing internal ribosome entry site (IRES) and blasticidin deaminase gene was excised from pMX-hor and subcloned into the same enzymatic site of pMX, generating pMRX-IRE5-bsr. The cDNA fragment of Fn14 was amplified by PCR using 290T cell-derived cDNAs as a template. The entire coding region of Fn14 cDNA was inserted into pCDNA3 (Invitrogen), pMRX-IRE5-puro (14), or pMRX-IRE5-bsr. The resultant plasmids were analyzed on FACSCalibur, and the data were analyzed by using the CellQuest program.

**Preparation of Whole Cell Extracts and Immunoblotting—** Cells were suspended in RIPA buffer (20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS) supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 100 μM Na<sub>3</sub>VO<sub>4</sub>, and 20 μM β-glycophosphatase. Extracts were cleared by centrifugation. Whole cell extracts (10 μg) were fractionated on 10–15% SDS-polyacrylamide gels and transferred onto Immobilon membranes (Millipore), and blots were revealed with an enhanced chemiluminescence detection system (ECL, Amersham Biosciences). All the experiments were carried out at least twice. The results were essentially reproducible.

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—** Cells were suspended in hypotonic buffer (10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 0.1 mM EDTA) supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 100 μM Na<sub>3</sub>VO<sub>4</sub>, and 20 μM β-glycerophosphate. After a 10-min incubation at 4 °C, Nonidet P-40 was added to 1%. The nuclear pellet was washed with hypotonic buffer and resuspended in extraction buffer (50 mM HEPES (pH 7.8), 50 mM KCl, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM phenylmethylosulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 2.5% glycerol). After a 30-min incubation at 4 °C with occasional agitation, DNA pellets were eliminated by centrifugation. The supernatant was recovered as nuclear extract. Nuclear extracts (5 μg) were incubated in 20 μl of binding buffer (10 mM HEPES (pH 7.8), 100 mM NaCl, 1 mM EDTA, 2.5% glycerol), 1 μg of poly[dI-C], and 0.6 ng of 32P-labeled β probe derived from the H-2K<sup>B</sup> promoter (39) or 32P-labeled Oct-1 probe (40) and incubated for 30 min at room temperature. For supershift assays, nuclear extracts were incubated with specific antibodies for 30 min on ice before incubation with the labeled probe. Samples were run on a 5% polyacrylamide gel containing 2.5% glycerol in 0.5× TBE and run as described above. All the experiments were carried out at least twice. The results were essentially reproducible.

**RESULTS**

**Identification of TWEAK as an Inducer of Long Lasting NF-κB Activation—** We sought to identify cDNAs whose expression leads to constitutive NF-κB activation in rat fibroblasts. For this purpose, we used a subline of Rat-1, B5, which stably expresses the blasticidin deaminase gene under the control of an NF-κB-dependent promoter. B5 cells were infected with retroviruses expressing a cDNA library prepared from Rat-1 and then subjected to a lethal selection with blasticidin S 36 h after infection. One of the isolated cell clones, 3-36, was found to exhibit constitutive NF-κB activity (Fig. 1A) and express TWEAK cDNA in the sense orientation. Retroviral expression of the recovered TWEAK cDNA in B5 cells indeed reproduced constitutive NF-κB activation in B5 cells, establishing the NF-κB-activating property of TWEAK. We next examined if addition of recombinant CD8-TWEAK fusion protein could also induce long-lasting NF-κB activation. Flow cytometric analysis revealed that Rat-1 cells could capture human CD8-TWEAK on their cell surface (Fig. 1B) and that both Rat-1 and B5 cells expressed Fn14, the TWEAK receptor (Fig. 1C). NF-κB DNA-binding complexes appeared 0.5 h after stimulation with CD8-TWEAK and lasted at least for 24 h with an apparent transi-
Fig. 1. TWEAK induces long lasting NF-κB activation. A, nuclear extracts were prepared from untreated B5 cells, an isolated cell clone 3-36 expressing a proviral form of TWEAK cDNA, and a pool of B5 cells established after infection with retrovirus expressing the cloned TWEAK cDNA. The extracts were incubated with 32P-labeled NF-κB probe or Oct-1 probe and subjected to EMSA. B, binding of CD8-TWEAK to Rat-1 cells was assessed by cell surface staining. Cells were incubated with phosphate-buffered saline (filled histogram) or CD8-TWEAK (open histogram) followed by PE-labeled anti-CD8 antibody. C, Fn14 expression on B5 and Rat-1 cells was assessed by cell surface staining with IgA (isotype control; filled histogram) or ITEM-2 (anti-Fn14 monoclonal antibody; open histogram). D, nuclear extracts were prepared from Rat-1 cells stimulated with CD8-TWEAK (100 ng/ml) or TNF-α (10 ng/ml) for the indicated periods. The extracts were incubated with 32P-labeled NF-κB or Oct-1 probe and subjected to EMSA. E, Rat-1 cells were stimulated with CD8-TWEAK (100 ng/ml) for 24 h in the presence of IgG (isotype control; 5 μg/ml) or CARL-1 (neutralizing antibody against TWEAK; 5 μg/ml). After the stimulation, nuclear extracts were prepared and subjected to EMSA. F, supershift assay. The nuclear extract of Rat-1 cells stimulated with CD8-TWEAK for 24 h was incubated with pre-immune (PI), anti-RelA, anti-RelB, or anti-p50 serum and then subjected to EMSA. G, Rat-1 cells were pre-treated for 30 min with Me2SO (Control; Ctl), MG132 (20 μM), or cycloheximide (CHX; 20 μg/ml) and then stimulated with CD8-TWEAK (100 ng/ml) for 8 h. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with antibodies to p100 or p52 or anti-actin antibody.
tion of the DNA-NF-κB complexes (Fig. 1D). The long lasting NF-κB DNA binding activity disappeared in the presence of CARL-1, a neutralizing antibody against human TWEAK (Fig. 1E), verifying that this NF-κB activation is TWEAK-dependent. In contrast, TNF-α induced NF-κB DNA binding activity only transiently, which decreased by 8 h after stimulation (Fig. 1D). To determine the subunit composition of the DNA-binding complexes induced 24 h after stimulation with TWEAK, we performed supershift assays using specific antibodies against RelA, RelB, or p50. Interestingly, the DNA-binding complexes contained RelB as well as p50 (Fig. 1F), but not RelA, at this time point. We were unable to examine participation of p52 in the DNA-binding complexes, because an antibody capable of supershifting rat p52 was not available.

**TWEAK Induces NF-κB2 p100 Processing**—RelB-containing DNA-binding complexes prompted us to examine if TWEAK induced p100 processing, because stimulation of LTβR or CD40 had been reported to induce RelB-containing DNA-binding complexes accompanied by p100 processing. As shown in Fig. 1G, TWEAK induced an obvious increase in p52 expression and reduction of p100 in Rat-1 cells 8 h after stimulation but failed to do so in the presence of MG132, a proteasome inhibitor, indicating that TWEAK induced proteasome-dependent processing of p100 to p52. Like the BAFF-R-, LTβR-, or CD40-mediated p100 processing, TWEAK-induced p100 processing was severely impaired by a protein synthesis inhibitor cycloheximide, indicating that *de novo* protein synthesis or a labile protein is required for TWEAK-induced p100 processing.

**TWEAK Activates Two Separate NF-κB Signaling Pathways**—Since prior studies revealed that NEMO was not required for p100 processing induced by BAFF-R or LTβR, we assessed the requirement of NEMO for TWEAK-induced NF-κB activation using NEMO-deficient 5R cells expressing the endogenous Fn14 (Fig. 2A). As shown Fig. 2, B and C, TWEAK induced p100 processing at 8 h post-stimulation, but not IκBα phosphorylation at 15 min, in 5R cells. At 0.5 h post-TWEAK stimulation, no appreciable DNA binding activity was observed in 5R cells, whereas a slowly migrating DNA-binding complex appeared in Rat-1 cells (Fig. 2D). Supershift assays revealed that this complex contained RelA and p50 (Fig. 2E). On the other hand, at 8 h post-TWEAK stimulation, similar NF-κB DNA-binding complexes containing RelB and p50 were induced regardless of the presence or absence of NEMO (Fig. 2, D and E). We were unable to examine p52 in these binding complexes due to poor supershifting of rat p52 by antibodies used in the assay. We therefore used mouse NIH3T3 cells that expressed Fn14 (Fig. 2F). As expected, TWEAK induced p100 processing (Fig. 2G) and NF-κB DNA-binding complexes containing p52 (Fig. 2H).

**IKK1 and NIK, but Not IKK2, Mediate TWEAK-induced p100 Processing**—The results that TWEAK activated NF-κB independently of NEMO raised a possibility that TWEAK might induce p100 processing through pathways that did not involve the IKK complex. To determine the requirement of IKK for TWEAK-induced p100 processing, we used MEFs, which lack IKK1 or IKK2. Because the cell surface expression levels of endogenous Fn14 in MEFs were quite low, we established pools of MEFs that expressed retrovirally transduced human Fn14 to similar levels (Fig. 3A). Western blot analyses revealed that TWEAK induced p100 processing in wild-type or IKK2−/− MEFs but not in IKK1−/− MEFs (Fig. 3B). In addition, TWEAK failed to induce p100 processing in NIKabl/aabl MEFs that did not express functional NIK (Fig. 3B). By contrast, TWEAK induced IκBα phosphorylation in wild-type or NIKabl/aabl MEFs, but not in IKK1−/− or IKK2−/− MEFs (Fig. 3C).

**Fn14 Regulates IκBα Phosphorylation and p100 Processing via TRAF-binding Motif**—Previous studies (27, 31) showed that the cytoplasmic domain of Fn14 bound to TRAF1, TRAF2, TRAF5, and TRAF6, but not to TRAF4 or TRAF6. Alkaline substitutions for the TRAF-binding motif (PIEBE), which had been reported to disrupt the interaction of Fn14 with the TRAF proteins (31), did not affect the cell surface expression of Fn14 (Fig. 4, A and B) but resulted in a complete loss of its ability to phosphorylate IκBα and process p100 in response to TWEAK (Fig. 4C). These results indicated that this TRAF-binding motif was essential for NF-κB activation mediated by Fn14.

**TRAF Proteins Regulate TWEAK-induced IκBα Phosphorylation and p100 Processing**—Because TRAF2 and TRAF5 were known to mediate NF-κB activation by members of the TNF superfamily, we examined whether these TRAF proteins were required for TWEAK-induced NF-κB activation. Fn14 was expressed by retroviral infection of TRAF2−/−/− MEFs to ensure sufficient expression of Fn14 (Fig. 4D). Neither TWEAK-induced p100 processing nor IκBα phosphorylation was detected in TRAF2−/−/− MEFs (Figs. 4, E and F). To confirm that the defect of NF-κB activation in TRAF2−/−/− MEFs was due to the disruption of TRAF2 and TRAF5, we tested whether TWEAK-induced NF-κB activation could be restored by TRAF expression in these cells. Retroviral expression of TRAF2 in TRAF2−/−/− MEFs expressing Fn14 substantially restored TWEAK-induced processing of p100 and phosphorylation of IκBα (Fig. 4, G–I), whereas TRAF5 expression partially improved these TWEAK-induced events. Collectively, our results demonstrate essential roles for TRAF2 and TRAF5 in the canonical and non-canonical NF-κB signaling pathways triggered by TWEAK.

**DISCUSSION**

Our present study revealed that TWEAK initiated two differentially regulated NF-κB signaling pathways. TWEAK rapidly induced IκBα phosphorylation and the RelA-containing DNA-binding complexes in a manner dependent on components of the canonical IKK complex. By 8 h post-stimulation, TWEAK induced the proteasome-dependent processing of p100 and the DNA-binding complexes containing p50 and RelB regardless of NEMO expression. This processing proceeded in the absence of IKK2 but not in the absence of IKK1. Moreover, TWEAK induced the rapid IκBα phosphorylation but not the subsequent p100 processing in NIKabl/aabl MEFs, suggesting that functional NIK activity is involved in the latter process. Regarding NIK, similar results were reported for CD40 and LTβR (12, 15). Thus, the delayed NF-κB activation by TWEAK is consistent with the recently identified non-canonical NF-κB activation. TWEAK failed to phosphorylate IκBα not only in 5R or IKK2−/− cells but also in IKK1−/− cells at 15 min post-stimulation. Similar IKK1 requirement for IκBα phosphorylation was reported in LTβR signaling, where engagement of LTβR by agonistic antibody failed to induce the IκBα phosphorylation (41) and the RelA-containing signaling components (15) in IKK1−/− MEFs.

Recent studies (24–25, 42) showing that TWEAK induced cell death of a certain tumor cell line via autocrine production of TNF-α raised a possibility that TNF-α might be involved in TWEAK-induced NF-κB activation. However, we and others (1–5, 8–9, 15) showed previously that TNF-α selectively initiated the NEMO-dependent canonical pathway and induced the RelA-containing DNA-binding complexes, whereas TWEAK was able to activate NF-κB independently of NEMO and induce the RelB-containing DNA-binding complexes as shown in this study. Moreover, ectopic expression of Fn14, but not of TNFRI, induced NF-κB-dependent transcription in NEMO-deficient 5R cells (data not shown). These observations strongly suggest
that NF-κB activation by TWEAK is not mediated by TNF-α and that TWEAK directly initiates both the canonical and non-canonical NF-κB activation pathways via Fn14.

The TRAF family proteins are critical mediators of NF-κB signaling pathways initiated by members of the TNF receptor superfamily. We showed previously (34) that TRAF2 and TRAF5 played critical roles for TNF-α-induced IκBα regulation, and others (12) showed that a point mutation in the TRAF2/3-binding motif of CD40 severely impaired its ability to process p100 and that overexpression of a dominant-negative form of TRAF2 inhibited CD40-mediated p100-processing, suggesting that TRAF proteins were involved in this process. The TRAF-binding motif found in Fn14 was shown to bind to TRAF2 and TRAF5 (27, 31). It was also reported that a mutant Fn14 lacking the TRAF-binding motif failed to induce NF-κB-dependent reporter gene activation (31). However, roles of this TRAF-binding motif in IκBα or p100 regulation have not been explored. Our present study revealed that the TRAF-binding motif of Fn14 was required for both IκBα phosphorylation and p100 processing. We next examined whether TRAF2 and TRAF5 were required for the regulation of IκBα and p100 by TWEAK. We used MEFs deficient in both TRAF2 and TRAF5, and we provided genetic evidence that TWEAK-induced IκBα phosphorylation and p100 processing were both severely impaired in TRAF2−/−TRAF5−/− MEFs. Reconstitution with TRAF2 restored TWEAK-induced p52 generation and IκBα phospho-
rylation in TRAF2−/− MEFs, whereas TRAF5 expression restored them minimally. These results suggest that TRAF2 plays an important role in the regulation of TWEAK-induced NF-κB activation pathways and that the contribution of TRAF5 is rather limited, at least in fibroblasts. Prior studies on LTβR or CD40 showed that mutant LTβR and CD40 lacking the TRAF-binding motif induced weak NF-κB activation (12, 43), leaving a possibility that certain molecule(s) other than the TRAF family members participate in regulation of IκB proteins. However, our present study indicated that the contribution of such molecule(s) to TWEAK-induced NF-κB signaling pathway in fibroblasts was quite low if any. It would be interesting to examine if other TNF receptor family proteins such as LTβR, BAFF-R, or CD40 trigger the p100 processing in TRAF2−/− MEFs.

We isolated by expression cloning the TWEAK cDNA from a cell clone that acquired constitutive NF-κB activity following infection with retroviruses expressing a cDNA library. Subsequently, we demonstrated that recombinant TWEAK could trigger both the canonical and non-canonical pathways of NF-κB activation in wild-type cells. Through this expression cloning, we also isolated cDNAs encoding LTβR, BAFF-R, or NIK, both of which had been implicated in the non-canonical pathway of

* T. Saitoh and S. Yamaoka, unpublished observations.

Fig. 3. IKK1 and functional NIK activity, but not IKK2, are required for TWEAK-induced p100 processing. A, cell surface staining of Fn14 on MEFs infected with pMRX-IRES-puro (empty vector; EV) or pMRX-Fn14-puro (Fn14). Cells were stained with control IgA (filled histogram) or ITEM-2 (open histogram). B, cells were stimulated with CD8-TWEAK (100 ng/ml) for 8 h. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with anti-p52 or anti-actin antibody. C, cells were stimulated with CD8-TWEAK (100 ng/ml) for 15 min. Whole-cell lysates were subjected to SDS-PAGE and immunoblotting with anti-p-IκBα or anti-actin antibody.
**Fig. 4.** TRAF proteins are involved in TWEAK/Fn14-mediated NF-κB signaling pathways. 

**A**, structure of A5-Fn14. Five amino acids in the TRAF-binding motif (PIEET) were substituted to alanine residues. **B**, cell surface expression of Fn14. Wild-type MEFs were transfected with pcDNA3 (empty vector, EV), pcDNA3-WT-Fn14, or pcDNA3-A5-Fn14. Cells were stained with control IgA (filled histogram) or ITEM-2 (open histogram). **C**, wild-type MEFs were transfected with pcDNA3, pcDNA3-Fn14, or pcDNA3-A5-Fn14. Thirty six hours after transfection, cells were stimulated with CD8-TWEAK (100 ng/ml) for the indicated periods. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with anti-p52, anti-p-IκBα, or anti-actin antibody. **D**, TRAF2-/- MEFs were infected with pMRX-IRES-puro (empty vector; EV) or pMRX-Fn14-puro (Fn14). The infected cells were stained with control IgA (filled histogram) or ITEM-2 (open histogram). **E**, the infected cells were stimulated with CD8-TWEAK (100 ng/ml) for 8 h. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with anti-p52 or anti-actin antibody. **F**, the infected cells were stimulated with CD8-TWEAK (100 ng/ml) for 15 min. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with anti-p-IκBα or anti-actin antibody. **G**, TRAF2-/- MEFs were infected with pMRX-Fn14-bsr and selected with blasticidin S. TRAF2-/- MEFs stably expressing human Fn14 were further infected with pMX-puro (empty vector; EV), pMX-FLAG-TRAF5 (TRAF5) and selected with puromycin. Drugs-resistant cells were stained with control IgA (filled histogram) or ITEM-2 (open histogram). **H**, cells established in G were stimulated with CD8-TWEAK (100 ng/ml) for 8 h. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with anti-p52, anti-FLAG, or anti-actin antibody. **I**, cells were stimulated with CD8-TWEAK (100 ng/ml) for 15 min. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with anti-p-IκBα, anti-FLAG, or anti-actin antibody.
NF-κB activation (13, 18, 22). These observations raise an intriguing possibility that the non-canonical pathway plays an important role in constitutive NF-κB activation. Indeed, a variety of tumor cells with constitutive NF-κB activity as well as mature B cells, differentiated macrophages, and dendritic cells have been reported to express p52 or RelB (7, 19, 44–49). Moreover, viral regulatory proteins, such as Tax of human T cell leukemia virus type 1 and latent membrane protein 1 of Epstein-Barr virus, induce p100 processing or p52 expression. Therefore, viral regulatory proteins, such as Tax of human T cell leukemia virus type 1 and latent membrane protein 1 of Epstein-Barr virus, induce p100 processing or p52 expression to persistently activate NF-κB (50–51). In these situations, disrupting the IκB function of p100 is likely important for maintenance of the NF-κB activity, but expression of a set of genes regulated by RelB may also contribute to the biological phenotypes.

In the present study, we demonstrated that TWEAK initiated both the canonical and non-canonical pathways to induce long lasting NF-κB activation. However, patho-physiological roles of TWEAK/Fn14 signaling remain to be studied. Previous reports (52, 53) showed that expression of Fn14 mRNA was up-regulated in regenerating liver, hepatocellular carcinoma, and glioma cells. Elevated NF-κB activity was observed in these carcinoma cells, and such activity was reported to be important for their survival (54–56). In addition, we found that NF-κB DNA binding activity containing p52 in a certain hepatocellular carcinoma cell line that aberrantly expressed Fn14. Thus, it is interesting to examine how TWEAK/Fn14 signaling contributes to the constitutive NF-κB activity in tumors and hence to the oncogenic processes.

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