Regulation of the NF-κB-inducing Kinase by Tumor Necrosis Factor Receptor-associated Factor 3-induced Degradation*

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The NF-κB family of transcription factors plays a pivotal role in regulation of diverse biological processes, including immune responses, cell growth, and apoptosis. Activation of NF-κB is mediated by both canonical and noncanonical signaling pathways. Although the canonical pathway has been extensively studied, the mechanism mediating the noncanonical pathway is still poorly understood. Recent studies have identified the NF-κB-inducing kinase (NIK) as a key component of the noncanonical pathway of NF-κB activation; however, how the signaling function of NIK is regulated remains unknown. We report here that one important mechanism of NIK regulation is through its dynamic interaction with the tumor necrosis factor receptor-associated factor 3 (TRAF3). TRAF3 physically associates with NIK via a specific recruitment motif located in the N-terminal region of NIK; this molecular interaction appears to target NIK for degradation by the proteasome. Interestingly, induction of noncanonical NF-κB signaling by extracellular signals involves degradation of TRAF3 and the concomitant enhancement of NIK expression. These results suggest that induction of noncanonical NF-κB signaling may involve the rescue of NIK from TRAF3-mediated negative regulation.

The transcription factor NF-κB regulates genes involved in diverse biological processes, such as immune and inflammatory responses, cell growth, and apoptosis (1–3). NF-κB represents a family of dimeric DNA-binding proteins, composed of RelA, RELB, c-Rel, NF-κB1 (or p50), and NF-κB2 (or p52) (4). NF-κB1 and NF-κB2 are produced as inactive precursors, p105 and p100, which undergo proteasome-mediated processing to generate the mature proteins, p50 and p52, respectively (4). Although various NF-κB complexes have been identified, the predominant forms of NF-κB are the heterodimers formed between the Rel proteins and p50 or p52. The NF-κB dimers are normally sequestered in the cytoplasm as inactive complexes with a family of ankyrin repeat-containing inhibitors, including IkBα and related proteins (5). Activation of NF-κB can be stimulated by a large variety of cellular stimuli, such as cytokines, mitogens, DNA-damaging agents, and microbial components (6). The canonical pathway of NF-κB activation is mediated by a large IκB kinase (IKK)1 complex, composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit termed IκKγ (also named NEMO, IKKAP1, or FIP-3) (6). Upon activation by the various NF-κB stimuli, IKK phosphorylates IκBα, triggering its ubiquitination and subsequent degradation by the proteasome, which results in rapid and transient nuclear translocation of the active NF-κB dimers.

Another mechanism of NF-κB regulation is through proteolytic processing of p105 and p100. In addition to serving as the precursors of p50 and p52, p105 and p100 function as IκB-like molecules (7–9). The processing of these precursor proteins not only leads to generation of p50 and p52 but also results in disruption of the IκB-like function of these precursor proteins. Recent studies suggest that although the processing of p105 is largely constitutive and cotranslational (10), the processing of p100 is tightly regulated by both positive and negative mechanisms (11). Induction of p100 processing is mediated by a noncanonical NF-κB signaling pathway that relies on the NF-κB-inducing kinase (NIK) (11) as well as its downstream kinase IκKα (12). This novel pathway of NF-κB activation does not require IKKβ and IκKγ (12–14), key components of the canonical NF-κB signaling pathway (15). In addition to producing p52, the processing of p100 is required for liberating RelB, allowing this specific NF-κB member to move to the nucleus and exert its gene regulation function (16–18). Genetic evidence suggests that the noncanonical NF-κB signaling pathway plays a key regulatory role in maturation of B cells and development of lymphoid organs (12, 19–21). Consistently, this novel pathway of NF-κB activation is regulated by a subset of tumor necrosis factor receptor (TNFR) family members known to be important for B-cell function and lymphoid organogenesis, including Baff receptor (BaffR), CD40, and lymphotoxin β receptor (14, 17, 22, 23).

How the different receptors mediate activation of the noncanonical NF-κB signaling pathway remains unclear. The TNFR-associated factor (TRAF) proteins may play an important role, since they are known to be recruited to various TNFR family members (24). Interestingly, a common TRAF molecule recruited by the different noncanonical NF-κB-stimulating receptors (BaffR, CD40, and lymphotoxin β receptor) is TRAF3 (25–27), although the role of this adaptor protein in regulation of

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1 The abbreviations used are: IKK, IκB kinase; NIK, NF-κB inducing kinase; TRAF, tumor necrosis factor receptor-associated factor; TNFR, tumor necrosis factor receptor; BaffR, Baff receptor; RNAI, RNA interference; siRNA, small inhibitory RNAs; GFP, green fluorescence protein; IP, immunoprecipitation; IB, immunoblotting; HA, hemagglutinin; E3, ubiquitin-protein isopeptide ligase.
the noncanonical NF-κB pathway remains unclear. In the present
study, we show that TRAF3 is a negative regulator of NIK, a
key signaling molecule involved in noncanonical NF-κB acti-
vation. TRAF3 physically interacts with NIK and targets NIK
for degradation by the proteasome. Remarkably, the receptor-
mediated activation of noncanonical NF-κB signaling is asso-
ciated with persistent degradation of TRAF3 and the marked
elevation of NIK expression. These findings suggest that induc-
tion of the noncanonical NF-κB signaling involves rescue of
NIK from TRAF3-mediated destruction.

MATERIALS AND METHODS

Plasmid Constructs—HA-ubiquitin has been described previously
(11, 13). HA-NIK was created by cloning the human NIK cDNA,
together with a C-terminal HA tag, into the pCMV4 expression vector
(28). Truncation mutants of NIK were generated by PCR and design-
ated by the specific amino acid residues retained in the mutant
proteins. For example, NIK(152–947) contains the region from amino acid
152 to 947. NIK mutants harboring internal deletions were produced by
site-directed mutagenesis (Stratagene) using wild type NIK expression
vector as template. To generate the HA-tagged TRAF3s, human TRAF1
and TRAF3 and murine TF2α cDNAs were cloned by reverse tran-
scriptase-PCR and inserted into the psKNA-HA vector (29) downstream of
the HA-Tag. Deletion mutants of luciferase were created by PCR. The
retroviral expression vectors encoding NIK, NIK(78–84), and TRAF3 were
constructed by inserting the corresponding cDNA into the PSXLSN retroviral vector
(provided by Dr. Inder M. Verma (30)). The expression vector encoding
human p100 was provided by Dr. Warren C. Greene.

Antibodies and Other Reagents—The antibodies for NIK (H248),
TRAF3 (H122), horseradish peroxidase-conjugated TRAF3, TRAF2 (C-
20), tubulin (TU-02), and RelB (C-19) were purchased from Santa Cruz
Biotechnology, Inc. Horseradish peroxidase-conjugated HA monochonocal
antibody (3F10) was from Roche Applied Science. Anti-mouse CD40
antibody was purchased from PharMingen. The anti-p100 antibody was
kindly provided by Dr. Warner C. Greene. Recombinant IKKα was a gift
from Dr. Michael Karin. Human recombinant Baff was purchased from
BIOSOURCE. The proteasome inhibitor MG132 was purchased from Calbiochem and used at a dose of 25 μM.

Yeast Two-hybrid Screening—Yeast two-hybrid screening was per-
formed using the MATCHMAKER LexA two-hybrid system (Clontech).
A cDNA fragment encoding the N-terminal 710 amino acids of NIK
was cloned into the pLexA vector downstream of the DNA binding domain
of LexA to generate the pLexA-NIK-(1–710) bait. This bait was used to
screen a Jurkat T cell library constructed using pB42AD vector (human
leukemia MATCHMAKER LexA cDNA library, Clontech) following the
manufacturer's instructions. Putative positive clones were retrans-
fected into LexA yeast strain with pLexA-NIK-(1–710) or a nontargeting
control bait, pLexA-lamin. After this step of false positive elimination, specific
clones were subjected to DNA sequencing and subsequent BLAST
search analyses.

Cell Culture, Transfection, and Retroviral Infection—M12.4.1 B cell
line (31) and its derivative stably transfected with hCDC40 (M12-hCDC40)
(32) were kindly provided by Dr. Guil A. Bishop. The M12-NIK cells
were created by infecting the M12-hCDC40 cells with the pCLXSN-NIK
retroviral vector as previously described (33), and bulk-infected cells
were used in the experiments. All of these B-cell lines were cultured in
RPMI medium supplemented with 10% fetal bovine serum, 2 mM l-
glutamine, antibiotics, and 10 μg/ml mercaptoethanol. The kidney car-
cinoma cell line 293 was cultured in Dulbecco's modified Eagle's medium
with antibiotics, and 10% heat-inactivated serum.

RNA Interference (RNAi)—Small inhibitory RNAs (siRNAs) specific
for human TRAF3 or the green fluorescence protein (GFP) were syn-
thesized by Dharmacon Research, Inc. (Lafayette, CO). The sequences
of the oligonucleotides are as follows: GFP siRNA, GUU GUC AGG UUC
CGA UGA UTT (antisense); TRAF3 siRNA, AGA GUC AGG UUC CGA
UAG UTT (antisense); TRAF3 siRNA, AGA GUC AGG UUC CGA UAG
UUT (sense); and AUC AUC GAC ACC UUG CAG UTT (antisense).

For siRNA delivery, 293 cells were seeded into 6-well plates to
around 70% confluence. After 12–16 h, the cells were transfected with
140 pmol of siRNA using Oligofectamine (Invitrogen). To maximize
the efficiency of RNAi, the transfection was repeated at 24 h following
the first transfection. The highest efficiency of RNAi was achieved when
the second transfection was carried out using LipofectAMINE 2000
(Invitrogen), in which the siRNA was mixed with 300 ng of carrier DNA
(pDNA). Accordingly, this strategy (an Oligofectamine transfection
followed by a LipofectAMINE transfection) was used in this study. At
about 48 h after the second transfection, the cells were collected for
preparation of protein lysates. The efficiency of RNAi-mediated gene
suppression was monitored by IB.

Reverse Transciptase-PCR—RNA was prepared from the indicated
cells and subjected to reverse transcripase-PCR analysis as described
(33) using the following primers: NIK, CAT GCA GAC AGG CAC CCC
AC (forward) and CGA ACA ATA TTT TGG TAG CAA GC (reverse);
glyceraldehyde-3-phosphate dehydrogenase, CTA CTG GCG CTG CCA
AGG C (forward) and GTG GGT GTT GTT GAA GTT (reverse).

RESULTS

Identification of TRAF3 as a Major NIK-associated Protein—We have previously shown that the N-terminal region of
NIK contains regulatory sequences that appear to inhibit the
function of NIK (28). This finding prompted us to investigate whether this negative regulatory region associates with any
cellular factors involved in NIK regulation. We performed
yeast two-hybrid screening using the N-terminal portion of
NIK (NIK1–710) as bait. A clone that was frequently isolated en-
coded the C-terminal portion of TRAF3. Although several other
TRAF members have been shown to interact with NIK (37, 38),
one of them were identified using this bait. This result was
consistent with the previous finding that the common TRAF-
binding site of NIK is located at its C terminus (37, 38), which
was missing in the bait used in our screening.

To confirm the NIK/TRAF3 physical interaction in mamma-
TRAF3, a panel of deletion mutants of NIK was subjected to interaction with TRAF3. NIK may contain a domain specifically mediating its strong major partner protein of NIK and that the N-terminal region of two-hybrid results, these findings suggest that TRAF3 is a and TRAF3 and demonstrated their strong interaction under expression, since the cell lysates contained even higher in vitro also performed binding assays using the coprecipitated TRAFs by IB using anti-HA antibody (Fig. 1A). The NIK protein complex was isolated by IP using anti-NIK followed by detecting 293 cells together with HA-tagged TRAF1, TRAF2, or TRAF3. The NIK/TRAF3 interaction was readily precipitated from the cell lysates by the anti-NIK antibody (top panel). The amounts of NIK and TRAFs in the cell lysates were monitored by direct IB using anti-HA (middle and lower panels). B, in vitro interaction between NIK and TRAF3. In vitro translated HA-NIK and HA-TRAF3 were incubated for 10 min on ice either alone (lanes 1 and 2) or together (lane 3) followed by IP using anti-NIK. The precipitated NIK and associated TRAF3 were detected by IB using horseradish peroxidase-anti-HA (upper panel). The NIK and TRAF3 proteins in the input materials were analyzed by direct IB (lower panel). C, the N-terminal region of NIK is required for its interaction with TRAF3. HA-tagged TRAF3 was transfected into 293 cells together with the indicated NIK mutants. The physical interaction of TRAF3 with the different NIK mutants was determined by co-IP assays (top panel) as described for A. The expression of NIK mutants and TRAF3 was monitored by direct IB using anti-HA (middle and bottom panels). D, schematic diagram of wild type (WT) form and deletion mutants of NIK. Their TRAF3 binding ability (+) or deficiency (−) is summarized based on the results presented in C.

Fig. 1. N-terminal region of NIK mediates stable association with TRAF3. A, NIK strongly interacts with TRAF3 but only weakly interacts with TRAF1 and TRAF2. HA-tagged NIK was transfected into 293 cells together with HA-tagged TRAF1, TRAF2, or TRAF3. The NIK protein complex was isolated by IP using anti-NIK followed by detecting the coprecipitated TRAFs by IB using anti-HA antibody (top panel). The amounts of NIK and TRAFs in the cell lysates were monitored by direct IB using anti-HA (middle and lower panels). B, in vitro interaction between NIK and TRAF3. In vitro translated HA-NIK and HA-TRAF3 were incubated for 10 min on ice either alone (lanes 1 and 2) or together (lane 3) followed by IP using anti-NIK. The precipitated NIK and associated TRAF3 were detected by IB using horseradish peroxidase-anti-HA (upper panel). The NIK and TRAF3 proteins in the input materials were analyzed by direct IB (lower panel). C, the N-terminal region of NIK is required for its interaction with TRAF3. HA-tagged TRAF3 was transfected into 293 cells together with the indicated NIK mutants. The physical interaction of TRAF3 with the different NIK mutants was determined by co-IP assays (top panel) as described for A. The expression of NIK mutants and TRAF3 was monitored by direct IB using anti-HA (middle and bottom panels). D, schematic diagram of wild type (WT) form and deletion mutants of NIK. Their TRAF3 binding ability (+) or deficiency (−) is summarized based on the results presented in C.

To map the domain of NIK mediating its interaction with TRAF3, a panel of deletion mutants of NIK was subjected to TRAF3-binding assays. Consistent with the yeast two-hybrid assays, two NIK mutants containing the N-terminal portion (1–736 and 1–710) were capable of TRAF3 association (Fig. 1C, top panel, lanes 4 and 5). On the other hand, a NIK mutant lacking the N-terminal 151 amino acids (positions 152–947) was largely defective in binding to TRAF3 (lane 3). A NIK mutant harboring an internal deletion of amino acids 30–120 (330–120) also exhibited a severe defect in TRAF3 association (lane 2). Additional truncation analyses revealed that the N-terminal 29 amino acids of NIK were dispensable for its TRAF3-binding function (Fig. 1C, upper panel, lanes 8–10). These results, which are summarized in Fig. 1D, suggest that the N-terminal TRAF3-binding domain of NIK is located between amino acids 30 and 120.

A Specific Sequence Motif of NIK Is Required for Its Strong Interaction with TRAF3—Prior studies suggest that TRAF3 binds to target proteins via a conserved sequence motif, PX-QX(S/T), which is also recognized by TRAF2 and certain other TRAF members (39). However, the TRAF3-binding domain of NIK does not contain such a motif. The preferential binding of NIK to a specific TRAF member, TRAF3, also suggests the involvement of a novel interaction mechanism.

To systematically define the TRAF3-binding motif of NIK, we generated a large panel of NIK mutants harboring various internal deletions in the TRAF3-binding domain (Fig. 2A). Interestingly, most of the sequences in this domain were dispensable for TRAF3 binding; these include those covering amino acids 31–50 (Fig. 2B, top panel, lane 3), 51–70 (lane 4), and 91–120 (lane 6). However, a region covering amino acids 71–90 was essential for the TRAF3-binding activity of NIK, since removal of this region largely abolished the NIK/TRAF3 interaction (lane 5). More detailed deletion analyses within this region revealed a sequence motif ISIIAQA (located between amino acids 78 and 84; see Fig. 2A) that was critical for the TRAF3-binding function of NIK. Deletion of this sequence motif generated a NIK mutant, NIK78–84, which was largely defective in TRAF3 binding (Fig. 2B, top panel, lane 9). On the other hand, removal of the flanking sequence downstream of this TRAF3-binding motif (amino acids 85–90) had no effect on the NIK/TRAF3 interaction (lane 10), although removal of the upstream flanking sequence (amino acids 71–77) caused a partial reduction (lane 8). These biochemical analyses, summarized in Fig. 2A, suggest that the strong interaction of NIK with TRAF3 requires a specific sequence motif (ISIIAQA) located in the N-terminal region of NIK.

We then determined the domain within TRAF3 that is required for its binding to NIK. As depicted in Fig. 2C, TRAF3 contains three major domains: a ring finger (RF) domain, a zinc finger (ZF) domain, and a TRAF domain. Co-IP assays were performed to detect the interaction between NIK and TRAF3 mutants lacking the different domains. Deletion of the ring finger domain of TRAF3 had no effect on its interaction with NIK (Fig. 2D, top panel, lane 2; also see summary in Fig. 2C). Further deletion of the zinc finger domain also did not abolish the NIK-binding function of TRAF3 (lane 3). On the other hand, the removal of 120 amino acids from the C terminus of TRAF3, which disrupted the TRAF domain, completely abolished its interaction with NIK (lane 4). Thus, the C-terminal TRAF domain of TRAF3 is required for its interaction with NIK.

Transfected TRAF3 Inhibits the Signaling Function of NIK—To investigate the functional significance of NIK/TRAF3 interaction, we examined the effect of TRAF3 on NIK-induced p100 processing. As expected (11), expression of NIK in 293 cells resulted in the induction of p100 processing, as demonstrated by generation of the processing product p52 (Fig. 3A, top panel, lane 2). Interestingly, this signaling function of NIK was inhibited by TRAF3 in a dose-dependent manner (lanes 3.
A C-terminal truncation mutant of TRAF3, TRAF-(1–423), failed to inhibit the NIK-mediated induction of p100 processing (lanes 6 and 7). Since the TRAF3-(1–423) is defective in NIK binding (see Fig. 2D), it raised the possibility that the TRAF3/NIK interaction is required for the negative regulation of NIK by TRAF3.

To further confirm this idea, we examined the inhibitory effect of TRAF3 on NIKΔ78–84, a NIK mutant lacking the TRAF3-binding motif (see Fig. 2, A and B). This NIK mutant retained its function in inducing p100 processing (Fig. 3B, upper panel, lane 5). In fact, it exhibited elevated function compared with the wild type NIK (compare lanes 2 and 5). Importantly, the signaling function of NIKΔ78–84 was no longer inhibited by TRAF3 (lanes 6 and 7). This result further suggests that the TRAF3/NIK physical interaction is required for TRAF3-mediated negative regulation of NIK. Parallel ki-
nase assays revealed that deletion of the TRAF3-binding sequence of NIK did not alter its catalytic activity. Both NIK and NIKΔ78–84 exhibited strong activity in autophosphorylation and phosphorylation of the target IKKα (Fig. 3C).

TRAF3/NIK Interaction Targets NIK Degradation through the Proteasome—In the transient transfection studies, we noticed that the level of NIK was reduced in cells cotransfected with TRAF3 (see Fig. 3, A and B, middle panel, lanes 3 and 4). This result was unlikely to be due to transfection variation, since it was observed in multiple experiments (data not shown). Further, the effect of TRAF3 on NIK expression appeared to require their physical interaction, since the C-terminal truncation mutant of TRAF3 (1–423) defective in NIK binding did not alter the level of NIK expression (Fig. 3A, middle panel, lanes 6 and 7). Similarly, the expression of NIKΔ78–84, a NIK mutant incapable of TRAF3 binding, was not affected by TRAF3 (Fig. 3B, middle panel, lanes 6 and 7). These results indicated that the TRAF3/NIK interaction might also serve as a mechanism to control the steady expression of NIK. To confirm this notion, we performed pulse chase assays to determine the stability of NIK. Indeed, when NIK was coexpressed with TRAF3 in 293 cells, the half-life of this kinase was greatly reduced (Fig. 4A).

We also noticed that the expression efficiency of NIKΔ78–84 was higher than that of the wild type NIK (data not shown) and had to adjust the amounts of expression vectors in the transfection experiments to achieve similar protein expression levels (see the legend to Fig. 3B). Although this difference could be due to the variation in transfection efficiency of different expression vectors, it also raised the possibility that the steady expression of NIK is affected by endogenous TRAF3. To minimize the effect of protein overexpression associated with transient transfection, we generated 293 cells stably expressing wild type NIK and NIKΔ78–84 using a retroviral gene delivery system. Due to the low copy number of retroviral insertion, the stably NIK-expressing cells were reinfected with a retroviral vector expressing NIK, NIK together with TRAF3, NIKΔ78–84, or NIKΔ78–84 together with TRAF3 were either not treated (−) or incubated for 2 h with 25 μM MG132 (+). Cell lysates were subjected to IB using anti-NIK (top panel), anti-TRAF3 (panel 2), or anti-tubulin (panel 3). The differential mobility of the exogenous and endogenous TRAF3 proteins was due to the presence of the HA tag in the exogenous TRAF3. RNA was extracted from the same cells and subjected to reverse transcriptase-PCR analysis to determine the NIK expression at the mRNA level. C, knockdown of endogenous TRAF3 by siRNA enhances NIK expression and p100 processing. The 293-NIK and 293-NIKΔ78–84 cells were transfected with siRNA for either the control GFP or TRAF3. Cell lysates were subjected to IB using the indicated antibodies to monitor expression of NIK (top panel), TRAF3 (panel 2), control tubulin (panel 3), or p100 and its processing product p52 (bottom panel). D, inhibition of NIK degradation by MG132 results in accumulation of NIK/TRAF3 complex. The indicated 293 infectants were either not treated (−) or incubated for 2 h with the proteasome inhibitor MG132. The interaction of NIK with endogenous TRAF3 was detected by co-IP (top panel), and the expression of NIK (middle panel) and TRAF3 (bottom panel) was detected by IB. E, polyubiquitination of wild type NIK but not NIKΔ78–84. 293 cells stably infected with NIK or NIKΔ78–84 were transiently transfected with HA-ubiquitin. The cells were incubated with MG132 (25 μM) for the indicated times followed by isolating NIK by IB using anti-NIK. The NIK/ubiquitin conjugates were detected by IB using anti-HA.

Fig. 4. TRAF3 induces NIK degradation through the ubiquitin/proteasome pathway. A, induction of NIK degradation by TRAF3. 293 cells were transfected with NIK or NIK plus TRAF3. The cells were pulse-labeled for 30 min with [35S]Met/Cys and then chased for the indicated times, and the radiolabeled NIK was detected by IP (upper panel). The intensities of the protein bands were quantitated by densitometry and are presented as percentages of that measured for the 0-h chased lanes (lane 1 for NIK-transfected cells and lane 4 for NIK/TRAf3-transfected cells). B, degradation of NIK but not NIKΔ78 by TRAF3 expression. 293 cells stably infected with NIK, NIK together with TRAF3, NIKΔ78–84, or NIKΔ78–84 together with TRAF3 were either not treated (−) or incubated for 2 h with 25 μM MG132 (+). Cell lysates were subjected to IB using anti-NIK (top panel), anti-TRAF3 (panel 2), or anti-tubulin (panel 3). The differential mobility of the exogenous and endogenous TRAF3 proteins was due to the presence of the HA tag in the exogenous TRAF3. RNA was extracted from the same cells and subjected to reverse transcriptase-PCR analysis to determine the NIK expression at the mRNA level.
encoding TRAF3. Indeed, the steady level of NIK was further reduced in cells co-infected with TRAF3 (Fig. 4B, top panel, compare lanes 1 and 3). In contrast, the exogenous TRAF3 did not affect the expression level of the NIK mutant (Δ78–84) defective in TRAF3 binding (compare lanes 5 and 7). These results further suggested that the steady level of NIK is negatively regulated by its interaction with TRAF3. To test whether the low steady level of NIK resulted from its degradation, we treated the cells with a proteasome inhibitor, MG132. Remarkably, the steady level of wild type NIK was greatly elevated following MG132 treatment (Fig. 4B, top panel, lanes 2 and 4). In contrast, the expression of NIKΔ78–84 was not significantly affected by MG132 (lanes 6 and 8). Taken together with the pulse-chase results (Fig. 4A), these data suggest the possibility that association of TRAF3 with NIK targets NIK for rapid degradation by the proteasome. Since NIK undergoes rapid turnover even in the absence of exogenous TRAF3, it is conceivable that the fate of NIK is also regulated by endogenous TRAF3.

To directly demonstrate that the instability of NIK is mediated by endogenous TRAF3, we took the RNAi approach to suppress the expression of endogenous TRAF3. siRNA for TRAF3 or the control GFP was transfected into cells expressing NIK or NIKΔ78–84. The TRAF3 siRNA dramatically suppressed the expression of TRAF3 (Fig. 4C, panel 2, lanes 2 and 4) but did not alter the expression level of tubulin (panel 3, lanes 2 and 4). Further, as expected, the GFP siRNA did not suppress the expression of TRAF3 (panel 2, lanes 1 and 3). Importantly, the suppression of TRAF3 expression by TRAF3 siRNA was associated with marked elevation in the level of wild type NIK (top panel, lane 2). In contrast, the TRAF3 suppression did not significantly alter the expression of NIKΔ78–84 (lane 4). Thus, binding of NIK to endogenous TRAF3 appears to target this kinase for degradation. Parallel IB assays revealed that the elevation of NIK expression in TRAF3 siRNA-treated cells was associated with enhanced processing of p100 (bottom panel, lane 2). On the other hand, the TRAF3 siRNA had no effect on p100 processing in cells expressing NIKΔ78–84, which already exhibited high levels of p100 processing (bottom panel) and NIK expression (top panel).

If the TRAF3/NIK physical interaction indeed serves as a trigger for NIK degradation, we should detect accumulation of the TRAF3-NIK complexes in cells incubated with proteasome inhibitors. We examined this idea by co-IP assays using 293 cells stably expressing NIK or NIKΔ78–84. In untreated cells, a low amount of endogenous TRAF3 was co-precipitated with NIK (Fig. 4D, top panel, lane 1). In contrast, no interaction was detected between TRAF3 and NIKΔ78–84 (lane 3), although this NIK mutant was expressed at higher levels than the wild type NIK (middle panel, compare lanes 1 and 3). Importantly, a considerably higher amount of TRAF3 was assembled into the NIK complex in cells incubated with the proteasome inhibitor MG132 (Fig. 4D, top panel, lane 2). The enhanced NIK/ TRAF3 association in MG132-treated cells was apparently due to the accumulation of NIK (middle panel, lane 2). On the other hand, the MG132 treatment did not enhance the expression of TRAF3 (bottom panel, lane 2). These results have several implications: 1) newly synthesized NIK rapidly associates with TRAF3; 2) NIK, but not TRAF3, is targeted for degradation upon their physical association; and 3) the intracellular amount of TRAF3 is in excess compared with NIK.

To examine whether the NIK degradation is mediated through its ubiquitination, we analyzed the polyubiquitination of wild type NIK and NIKΔ78–84 in cells treated with MG132. Indeed, when the degradation of NIK was inhibited by MG132, its ubiquitinated forms could be readily detected (Fig. 4E, lanes 1–3). Consistent with its inability to associate with TRAF3, the NIKΔ78–84 was not significantly ubiquitinated (lanes 4–6).

Noncanonical NF-κB Stimuli Induce Degradation of TRAF3 and Elevation of NIK Expression—Recent studies have shown that the noncanonical NF-κB signaling pathway can be activated by ligands for a subset of TNFR family members, including BaffR, CD40, and lymphotoxin β receptor (14, 17, 22, 23), although the underlying mechanisms remain unclear. We examined whether the noncanonical NF-κB signaling involves modulation of the TRAF3/NIK molecular interplay. For these studies, we employed a murine B-cell line, M12.4.1 (hereafter referred to as M12), which had been frequently used to study CD40 signaling (31, 32, 40, 41). Since both CD40 and BaffR are expressed on B cells, we reasoned that the M12 cells might serve as a convenient model for studying noncanonical NF-κB signaling. Indeed, stimulation of the M12 cells with either anti-CD40 or Baff resulted in potent processing of p100 (Fig. 5A, upper panel) as well as nuclear translocation of RelB (data not shown) (Fig. 5B, bottom panel). Interestingly, the noncanonical NF-κB signaling events were associated with persistent degradation of TRAF3 (Fig. 5A, lower panel). Time course studies showed that TRAF3 was largely depleted around 2 h after cellular stimulation by Baff or anti-CD40, although partial loss of TRAF3 was detectable at an earlier time point (1 h) (Fig. 5B, top panel). Parallel analysis of TRAF2 revealed that, as previously reported (42), this TRAF member was also degraded by the anti-CD40 signal (second panel, lanes 6–8). In sharp contrast, however, the fate of TRAF2 was not appreciably affected in Baff-stimulated cells (second panel, lanes 2–4). On the other hand, both Baff and anti-CD40 efficiently stimulated the nuclear translocation of RelB (bottom panel). Thus, degradation of TRAF3, but not that of TRAF2, is associated with noncanonical NF-κB activation. Since TRAF3 promotes NIK degradation, it is tempting to speculate that the CD40 and BaffR signals may rescue NIK from TRAF3-induced degradation.

Our initial IB analyses indicated that the level of endogenous NIK is extremely low, since no specific NIK protein was detected by various commercial anti-NIK antibodies (data not shown). To overcome this technical difficulty, we concentrated NIK by IP from the cell lysates before performing IB analyses. With this IP-coupled IB approach, we were able to detect a weak NIK band (Fig. 5C, top panel, lane 1). Interestingly, the level of NIK expression was significantly enhanced upon stimulation of the cells with anti-CD40 (lane 2) or Baff (lane 3) but not the canonical NF-κB inducer tumor necrosis factor-α (lane 4). To further confirm that the steady level of NIK could be induced by noncanonical NF-κB stimuli, we generated M12 B cells stably expressing exogenous NIK by retrovirus-based gene transfer. The steady level of exogenous NIK was also low, which required IP-coupled IB for detection (Fig. 5C, top panel, lane 5). More importantly, the exogenous NIK was also induced by the anti-CD40 and Baff signals and was readily precipitated by the anti-NIK antibody (lanes 6 and 7) but not a preimmune serum (lane 8). Further, the induction of NIK expression was associated with degradation of TRAF3 (Fig. 5C, middle panel).

The studies presented above strongly suggest that the endogenous NIK may be subject to the negative regulation by TRAF3. To examine this possibility, we incubated the M12 B cells with the proteasome inhibitor MG132. Indeed, significantly more endogenous NIK was detected in the MG132-treated cells (Fig. 5D, top panel, lane 2). We then investigated whether NIK and TRAF3 are associated under endogenous conditions and whether this molecular interaction precedes NIK degradation. Consistent with the low expression level of NIK, little TRAF3 was detected in the NIK immune complex in
untreated cells (Fig. 5D, middle panel, lane 1). However, inhibition of NIK degradation by MG132 resulted in accumulation of the NIK/TRA3 complex, as demonstrated by the coprecipitation of TRA3 with NIK (lane 2). Our attempt to perform pulse-chase assays proved to be technically difficult, since the level of endogenous NIK or stably infected NIK was too low to obtain efficient pulse labeling (data not shown). Nevertheless, these findings indicate that binding of TRA3 to NIK targets NIK for degradation by the proteasome and that the noncanonical NF-κB stimuli may prevent NIK degradation by eliminating TRA3.

**DISCUSSION**

NIK serves as a key component in a noncanonical NF-κB signaling pathway that targets the processing of NF-κB p100 and, in turn, the nuclear translocation of RelB-containing NF-κB dimers (43). How the signaling function of NIK is regulated has remained unclear. The results presented in this paper establish TRA3 as a negative regulator of NIK. TRA3 physically interacts with NIK via a novel sequence motif located at the N-terminal region of NIK. This molecular interaction may serve to prevent uncontrolled function of NIK. At least in transfected cells, TRA3 inhibits NIK-mediated induction of p100 processing. More importantly, the TRA3/NIK interaction targets NIK for continuous degradation by the proteasome, a mechanism that may contribute to the low intracellular level of NIK. We have demonstrated that the TRA3-mediated NIK destruction can be prevented by the noncanonical NF-κB stimuli, including anti-CD40 and Baff. This step of signaling seems to be achieved through signal-induced degradation of TRA3. These findings suggest a novel mechanistic mediation of NF-κB signaling, which involves the rescue of NIK from its TRA3-programmed destruction.

It remains to be further investigated how the catalytic activity of NIK is activated in response to receptor signals. It is possible that the activation of NIK involves both of its modifications by upstream signaling components (e.g., kinases) and its protection from TRA3-induced proteolysis. However, since the transfected NIK exhibits remarkably high constitutive activity in mammalian cells (11, 37, 44), it is also conceivable that the function of NIK may be primarily controlled by its level of expression. We have found that the preexisting level of NIK is extremely low and is only weakly detected by IP-coupled IB assays. When stably expressed in M12 B cells and 293 cells, the exogenous NIK is also maintained at a low level. At least in 293 cells, in which RNAi could be efficiently performed, the level of NIK can be markedly enhanced by RNAi-mediated suppression of endogenous TRA3 (Fig. 4C). This genetic evidence strongly indicates that the low intracellular level of NIK is due to its degradation targeted by its partner protein TRA3. In agreement with this model, induction of TRA3 degradation by noncanonical NF-κB stimuli is also associated with elevation of NIK expression (Fig. 5C).

The TRA3/NIK interplay seems to be reminiscent of the p53 regulation by its inhibitor Mdm-2 (45). It has been shown that newly synthesized p53 is quickly bound by Mdm-2 and targeted to the proteasome for degradation. In this case, Mdm-2 is known to function as a ubiquitin ligase (E3) that stimulates polyubiquitination of p53 (46). Mdm-2 belongs to a family of ubiquitin ligases that contain ring finger domains (47, 48). Notably, TRA3, as well as most other TRA3 members, also contain a ring finger. It is thus possible that TRA3 may induce NIK degradation through the ubiquitination pathway. We have found that NIK indeed undergoes polyubiquitination in MG132-treated cells (Fig. 4E). This modification of NIK requires its association with TRA3, since a NIK mutant lacking the TRA3-binding site (∆78–84) is not ubiquitinated (Fig. 4E). However, whereas these results suggest that the TRA3/NIK association triggers NIK ubiquitination, our preliminary
in vitro ubiquitination studies using purified TRAF3 did not reveal significant E3 activity (data not shown). Nevertheless, although this result suggests that TRAF3 itself may not be sufficient to act as a ubiquitin ligase, it does not exclude the possibility that this NIK-binding protein functions as a critical component of a ubiquitin ligase mediating NIK ubiquitination in vivo. Notwithstanding, the results presented in this paper establish TRAF3 as a negative regulator of NIK, mediating degradation of NIK through the proteasome pathway.

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