The non-canonical pathway based on processing of NF-κB2 precursor protein p100 to generate p52 plays a critical role in controlling B cell function and lymphoid organogenesis. Activation of this unique pathway by extracellular stimuli requires NF-κB-inducing kinase (NIK) and de novo protein synthesis. However, how NIK is regulated is largely unknown. Here, we systematically analyzed NIK expression at different levels in the presence or absence of different NF-κB stimuli. We found that NIK mRNA is relatively abundant and undergoes constitutive protein synthesis in resting B cells. However, NIK protein is undetectable. Interestingly, protein expression of NIK is steadily induced by B cell-activating factor or CD40 ligand, two major physiological inducers of p100 processing, but not by mitogen phorbol 12-myristate 13-acetate/ionicomycin or cytokine tumor necrosis factor α, two well known inducers of the canonical NF-κB signaling. Remarkably, both B cell-activating factor and CD40 ligand do not significantly induce expression of NIK at translational or transcriptional level but rather rescue the basally translated NIK protein from undergoing degradation. Furthermore, overexpressed or purified NIK protein triggers p100 processing in the presence of protein synthesis inhibitor. Taken together, these studies define one important mechanism of NIK regulation and the central role of NIK stabilization in the induction of p100 processing. These studies also provide the first evidence explaining why activation of the non-canonical NF-κB signaling is delayed and can be inhibited by protein synthesis inhibitor as well as why most classical NF-κB stimuli, including mitogens and tumor necrosis factor α, fail to induce p100 processing.

NIK-κB represents a collection of dimeric transcription factors that in mammals comprises five members: RelA (p65), RelB, c-Rel, NF-κB1 p50, and NF-κB2 p52 (1–3). Unlike the three Rel proteins, which are synthesized directly as mature proteins, p50 and p52 are generated by proteolytical processing from their large precursors, p105 and p100, respectively (4, 5). In resting cells, NF-κB dimers are sequestered in the cytoplasm as latent complexes with a family of ankyrin repeat domain-containing inhibitors called IκB proteins (1–3). Interestingly, both p105 and p100 contain ankyrin repeats at their C-terminal regions and function as IκB-like inhibitors of NF-κB (6, 7). Whereas the IκB degradation and p100 processing are inducible, the processing of p105 is constitutive (8, 9). Therefore, there are two major pathways leading to induction of NF-κB activation: the IκB degradation (canonical) and p100 processing (non-canonical) pathways (10).

The canonical NF-κB pathway is required for fundamental functions of various cells and can be rapidly and transiently activated by a plethora of substances such as mitogens, cytokines, and microbial components (11). These stimuli ultimately lead to activation of a specific IκB kinase (IKK) complex composed of two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit, IKKγ (NEMO) (10). Once activated, IKK phosphorylates specific serines within the IκB proteins, triggering their ubiquitination by the ubiquitin ligase β-TrCP and degradation by the 26 S proteasome, thus allowing the NF-κB dimers to move to the nucleus to induce gene expression (10).

Unlike IκB degradation, the activation of the non-canonical NF-κB pathway is strictly dependent on the IKKα and its activator, NF-κB-inducing kinase (NIK), but independent of IKKβ and IKKγ (9, 12, 13). Furthermore, this novel NF-κB pathway only occurs in certain cell types at certain stages and responds to very limited stimuli, such as lymphotoxin β (9, 14), B cell-activating factor (BAFF) (15, 16), and CD40 ligand (CD40L) (17). These specific stimuli activate NIK, which in turn activates IKKα and recruits it into p100 complex to phosphorylate p100 (18). The phosphorylation of p100 results in ubiquitination and subsequent processing of p100 mediated by the ubiquitin ligase β-TrCP and 26 S proteasome, respectively (9, 18–21).

Currently, the mechanism by which the physiological inducers activate NIK to trigger p100 processing remains largely unknown. Here, we have shown that BAFF- and CD40L-mediated NIK activation is through up-regulation of NIK protein level. In the resting B cells, NIK protein is undetectable, although NIK mRNA is relatively abundant and undergoes constitutive protein synthesis. The undetectable NIK protein is due to proteasome-mediated degradation. Although there is no contribution to NIK expression at transcriptional and translational levels, both BAFF and CD40L significantly up-regulate the level of NIK protein by rescuing the basally translated NIK proteins from undergoing degradation. Interestingly, overexpressed or purified NIK protein triggers p100 processing in the absence of additional stimuli and de novo protein synthesis. These results thus suggest that NIK protein stabilization functions as a switch for inducible processing of p100. These results also provide the first evidence for explaining why the non-canonical NF-κB pathway is delayed and can be inhibited by protein synthesis inhibitor.

MATERIALS AND METHODS

Expression Vectors and Antibodies and Other Reagents—Expression vectors encoding p100 and NIK have been described before (9). The
anti-NIK (H248) and anti-IKKα (H744) antibodies were from Santa Cruz Biotechnology, Inc. The antibody recognizing the N terminus of p100 (anti-p100N) was described previously (20, 21). The monoclonal antibody for human CD40 was prepared from a hybridoma (G28–5) provided by Dr. S. C. Sun. Tumor necrosis factor α (TNFα), cycloheximide (CHX), phosphorib 12-myrstate 13-acetate, and ionomycin were from Sigma. Human BAFF and MG132 were from Amgen Biologicals and Calbiochem, respectively.

Cell Culture and Transfection—Human B cell line Romas RG69 was a gift from Dr. L. Covey. Human embryonic kidney 293 cells were described before (12, 21). 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM l-glutamine, and Romas RG69 cells were cultured in RPMI supplemented with 10% fetal bovine serum and 2 mM l-glutamine. 293 cells were transfected with DEAE-Dextran as described before (22–24).

Immunoblotting (IB) and Immunoprecipitation (IP)—Whole-cell lysates and cytoplasmic lysates were prepared by lysing the cells in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% sodium-deoxycholate, 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and Buffer B (10 mM Hepes, pH 7.9, 10 mM KCl, 0.4% Nonidet P-40, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) supplemented with a protease inhibitor mixture, respectively. For IB assays, the whole-cell lysates (~10 μg for transfected 293 cells and ~30 μg for other cells) were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to IB using the indicated antibodies. For NIK IP assays, the cytoplasmic lysates (~1000 μg) were diluted to 1 ml using RIPA buffer, incubated with 5 μl of NIK antibodies for 1 h and with 30 μl protein-A-agarose for another 2 h at 4 °C. The agarose beads were washed three times with RIPA buffer, and the bound proteins were eluted by 2X SDS loading buffer and subjected to SDS-PAGE and IB analysis (20–23).

Cell Labeling and Pulse-Chase Assays—Transfected 293 cells were starved for 1 h in Dulbecco’s modified Eagle’s medium lacking methionine and cysteine, followed by metabolic labeling for 30 min with 350 μCi/ml [35S]methionine/[35S]cysteine. The pulse-labeled cells were chased for different time periods in complete Dulbecco’s modified Eagle’s medium supplemented with 10 mM cold methionine and lysed in RIPA buffer. The radiolabeled p100 and its processed product p52 were isolated by IP using anti-p100N antibodies, fractionated by SDS-PAGE, and visualized by autoradiography (9, 12, 21).

For the stability of endogenous NIK proteins, the Romas RG69 B cells were labeled for 2 h with [35S]methionine/cysteine in the presence or absence of BAFF or anti-CD40, followed by chasing in normal medium supplemented with 10 mM cold methionine. The radiolabeled NIK proteins were isolated by IP using anti-NIK antibodies, or the B cells were treated for 2 h with BAFF or anti-CD40 or phosphate-buffered saline (mock) and chased by directly adding cycloheximide (100 μg/ml) into medium. The NIK proteins were immunoprecipitated and detected by IB using anti-NIK antibodies.

In Vitro p100-processing Assays—The NIK proteins were generated in vitro with the TNT T7-coupled transcription/translation system (Promega) and further purified by NIK IP. The cell extracts from 293 cells transfected with p100 were prepared as described before (4). The immunopurified NIK proteins from in vitro translation and the 293 cell extracts were mixed in the presence or absence of 100 ng/μl CHX. The mixture then was incubated for 2 h at 30 °C in the presence of 0.5 mM ATP, 10 mM phosphocreatine, and 0.2 μg/μl creatine kinase for in vitro processing reaction, followed by IB using p100N antibodies.

Isolation of Polysomes and RNA from Romas RG69 Cells—Romas RG69 cells were treated for 2 h with anti-CD40 antibody (10 μg/ml) or BAFF (250 ng/ml) or left untreated. 20% of treated or untreated cells (2 × 10^6 cells/group) were pelleted and employed as a source for total RNA using TRIzol reagent (Invitrogen). The remaining cells were incubated with cycloheximide (100 μg/ml) for 15 min, followed by cytoplasmic extraction. The cytoplasmic extract was then loaded onto a linear 10–45% (w/w) sucrose gradient and centrifuged for 2.5 h at 36,000 rpm with a Beckman SW-41 rotor. After centrifugation, the gradient was fractionated and its absorbance at 254 nm was determined continuously by an Isco UA-5 monitor as described before (25). The polysome-containing fractions were pooled and subjected to phenol extraction. RNA was precipitated with ethanol and dissolved in diethyl pyrocarbonate-treated water.

Reverse Transcription (RT)-PCR and Real-time RT-PCR Analysis—2 μg of total RNA or RNA isolated from polysomes were reverse-transcribed for RT-PCR using the following primers: for NIK, forward 5′-CTTACAGCATGCACAGCCCTCCTCT, reverse 5′-GTGCCATGCGGCCGAGATGC; for glyceraldehydes-3-phosphate dehydrogenase, forward 5′-CAGTGGCATGCGCATCAG and reverse 5′-CCTACTCCTGGAGGCCCATG.

Real-time PCR assays were performed with an ABI Prism 7900HT sequence detection system using the SYBR Green PCR Core reagent (Applied Biosystems, Foster City, CA). Primers used for real-time PCR were: human NIK, forward 5′-CCACCTTTTCTGAGAGCAT, reverse 5′-CATTGTTGCCTGTAGCATGG; human p100, forward 5′-TGCCATGTTGGTCGACAGACAGCTCC; human β-actin, forward 5′-ATCAAGATCTTCAGCTCTCTTGC, reverse 5′-AGCGAGGCCGAGATGG; human 18 S RNA, forward 5′-AGGAATTTGACGAAGGAC, reverse 5′-GGACATCTAAGGGCCTACA.

RESULTS

The Dynamics of p100 Processing Triggered by Receptor Ligation Is Much Slower than That Induced by Downstream NIK—To investigate the mechanism by which receptor ligation activates downstream NIK to
p100 Processing Controlled by NIK Protein Stabilization

To test this possibility, we examined p100 processing in NIK-transfected cells in the presence or absence of CHX, a specific protein synthesis inhibitor. In sharp contrast to receptor ligation-mediated processing of p100 (Fig. 2A), NIK-induced p100 processing is insensitive to CHX (Fig. 2B, compare lane 4 with lane 5). To extend our studies, we also performed in vitro p100 processing assay using purified NIK protein. Consistently, purified NIK could also efficiently induce p100 processing in vitro even in the presence of CHX (Fig. 2C, lanes 3 and 4). Thus, NIK-mediated p100 processing does not require protein synthesis, supporting that NIK, but definitely not p100, is the newly synthesized protein required for processing of p100 induced by receptor ligation.

NIK Protein Expression Is Specifically Up-regulated by Receptor Ligation for p100 Processing and This Up-regulation Can Be Blocked by Protein Synthesis Inhibitor—If our hypothesis above is right, the expression of NIK should be very low in unstimulated cells and the receptor ligation for p100 processing would induce NIK protein expression. To confirm this, we examined the expression of endogenous NIK protein. Indeed, the level of NIK protein is extremely low, because we failed to detect NIK protein even by direct NIK IP assays (Fig. 3, upper panel). More importantly, the NIK protein was significantly increased upon stimulation of the cells with BAFF or CD40 antibody (Fig. 3A, upper panel, lanes 4 and 5). The expression of IKKα was also detected by direct IB. The asterisk indicates a nonspecific background band from IP. B, inhibition of NIK protein induction by protein synthesis inhibitors. B cells were either untreated (NT) or treated for 2 h with BAFF or anti-CD40 in the presence or absence of CHX, followed by IP-IB as described in panel A. The expression of p100 and its processed product p52 was also detected by IB using anti-p100N (lower panel). Again, the nonspecific background band from IP (NS) was also indicated (upper panel).

FIGURE 2. Protein synthesis inhibitor CHX inhibits p100 processing induced by receptor ligation but not that induced directly by NIK. A, inhibition of receptor ligation-induced p100 processing by CHX. B cells were treated with BAFF or anti-CD40 for 4 h in the presence or absence of CHX (100 ng/ml), followed by IB using anti-p100N. B, no effect of CHX on NIK-induced p100 processing. 293 cells were transfected with p100 in the presence or absence of NIK. The cells were pulse labeled for 30 min followed by 4 h of chase in the presence or absence of CHX or proteasome inhibitor MG132 (50 μM). C, p100 processing induced by NIK in vitro. In vitro translated (IVT) NIK proteins were further immunopurified, mixed with the cytoplasmic extracts of 293 cells transfected with p100 in the presence or absence of CHX. After 2 h of incubation at 30 °C, p100 processing was examined by IB.

FIGURE 3. Non-canonical NF-κB stimuli specifically induce expression of NIK proteins, an event that can be blocked by CHX. A, induction of NIK protein by receptor ligation. B cells were either untreated (NT) or treated with mitogens (10 ng/ml phorbol 12-myristate 13-acetate and 1 μM ionomycin), TNF-α (10 ng/ml), BAFF, or anti-CD40 for 2 h, followed by NIK IP. The enriched NIK proteins were detected by IB using anti-NIK antibodies. In vitro translated (IVT) NIK proteins were used as a positive control (lane 6). The expression of IKKα was also detected by direct IB. The asterisk indicates a nonspecific background band from IP. B, inhibition of NIK protein induction by protein synthesis inhibitors. B cells were either untreated (NT) or treated for 2 h with BAFF or anti-CD40 in the presence or absence of CHX, followed by IP-IB as described in panel A. The expression of p100 and its processed product p52 was also detected by IB using anti-p100N (lower panel). Again, the nonspecific background band from IP (NS) was also indicated (upper panel).
tion of RNA Transcription or Protein Translation of NIK—To further investigate the mechanism of NIK protein up-regulation, we performed real-time RT-PCR and polysome fractionation analysis to examine whether either BAFF or CD40 antibody treatment induces mRNA transcription and protein translation of NIK, respectively. As a control, the transcription and translation of p100 mRNA was also included. Interestingly, we found that CD40 antibody treatment, but not BAFF treatment, enhanced p100 mRNA and subsequent protein translation (Fig. 4, panels A and B, columns 4–6), which is consistent with the finding that CD40L can also activate canonical NF-κB to induce p100 expression whereas BAFF can only activate non-canonical, but not canonical, NF-κB (15). Unexpectedly, the NIK mRNA, like p100 mRNA, also showed constitutive translation (Fig. 4A, column 1). It was more surprising that BAFF treatment and CD40 antibody treatment hardly enhance NIK protein synthesis (Fig. 4A, columns 2 and 3). Consistently, our RT-PCR and real-time RT-PCR analyses indicated that the total mRNA level of NIK was relatively abundant and marginally affected by either BAFF or CD40 antibody treatment (Fig. 4B, columns 1–3). Based on the results above, we then postulated that the synthesized NIK proteins from the basal translation are immediately degraded and the ligation of BAFF receptor and CD40 stabilizes these newly synthesized NIK proteins. Indeed, we found that in the presence of MG132, a specific proteasome inhibitor, NIK protein could be detected even without stimulation by BAFF or CD40 antibody (Fig. 4C, upper panel, lane 2). However, this effect of MG132 could be blocked by the protein synthesis inhibitor CHX (Fig. 4C, upper panel, lane S). Consistent with
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our polysome fractionation analysis indicating that BAFF and CD40 antibody treatments do not contribute to NIK protein synthesis, neither BAFF nor CD40 antibody treatment further enhanced NIK protein level in the presence of MG132 (Fig. 4C, upper panel, lanes 3 and 4). Thus, ligation of BAFF receptor and CD40 rescued the basally translated NIK proteins from undergoing degradation, thereby triggering p100 processing.

To further substantiate our findings, we tried to compare the stability of NIK protein in the stimulated and unstimulated B cells by performing two different pulse-chase assays. Both pulse-chase assays indicated that the half-life of NIK in the presence of either BAFF or anti-CD40 antibodies was ~3 h (Fig. 5A, lanes 3–10, 5B, 5C, lanes 1–6, and 5D). However, we failed to examine the half-life of NIK in the unstimulated B cells because we were unable to obtain sufficient NIK for efficient detection or pulse labeling (Fig. 5A, lane 1, 5C, lane 7, and data not shown). In fact, we were even unable to obtain enough NIK proteins from B cells stably infected with NIK for pulse-chase assays (data not shown). Nevertheless, these results were highly consistent with our findings that NIK protein in the resting B cells was extremely low (Figs. 3 and 4). These results also suggested that the newly synthesized NIK protein in the resting B cells was rapidly degraded by the proteasome, whereas the BAFF or anti-CD40 treatment was to protect NIK from undergoing degradation.

DISCUSSION

It is clear that NIK is essential for non-canonical NF-κB signaling. However, how NIK is regulated remains largely unknown. In this study, we systematically analyzed NIK expression at different levels in the presence or absence of the non-canonical NF-κB stimuli BAFF or CD40L. In unstimulated cells, the expression level of NIK protein is extremely low; however, NIK expression is significantly up-regulated by BAFF and CD40L (Fig. 3A). The up-regulation of NIK protein is specific, because TNF and mitogens fail to induce NIK protein (Fig. 3A). Because TNF and mitogens also fail to induce p100 processing, although they efficiently activate IKKα (downstream kinase of NIK for p100 processing) (8, 12), these studies provide evidence why most of the canonical NF-κB stimuli including mitogens and TNFα fail to induce p100 processing, further suggesting that up-regulation of NIK proteins serves as a molecular switch for p100 processing. Indeed, transfected or purified NIK induces p100 processing in the absence of additional stimuli and de novo protein synthesis (Fig. 2, B and C).

Although the up-regulation of NIK protein and subsequent p100 processing by receptor ligation can be blocked by protein synthesis inhibitor (Fig. 2A and Fig. 3), these non-canonical NF-κB inducers apparently fail to enhance RNA transcription and protein translation of NIK (Fig. 4, A and B). In fact, NIK mRNA is relatively abundant and constitutively undergoes basal translation (Fig. 4, A and B). Thus, it seems that the role of the non-canonical NF-κB stimuli in p100 processing is to inhibit NIK degradation machinery so that the NIK proteins from basal translation gradually accumulate. Indeed, inhibition of the proteasome also efficiently up-regulates NIK protein level (Fig. 4C). These findings thus not only demonstrate one important mechanism of NIK regulation but also explain why the non-canonical NF-κB pathway is delayed and can be inhibited by protein synthesis inhibitor (Figs. 1 and 2A).

Our studies also rule out the possibility that de novo synthesis of p100 protein itself is required for its processing. First, transfected or purified NIK induces p100 processing in the absence of de novo protein synthesis, including de novo synthesis of p100 protein (Fig. 2, B and C). Second, stimuli that fail to up-regulate NIK protein also fail to induce p100 processing, though they can activate IKKα and induce p100 expression (12) (Fig. 3A). Third, p100 undergoes constitutive synthesis but no processing without specific stimuli. Last, BAFF induces p100 processing but cannot induce p100 synthesis (Fig. 4A). Although CD40 induces p100 protein expression via canonical NF-κB activation, the induced p100 is not required for, although it contributes to, CD40-induced p100 processing because CD40-induced p100 processing is independent of IKKγ, an essential component for canonical NF-κB activation and subsequent p100 induction. Together, these findings strongly suggest that NIK, but not p100, is the newly synthesized protein required for p100 processing, further substantiating our previous finding that p100 processing is post-translational (9).

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