Essential Role of IκB Kinase α in the Constitutive Processing of NF-κB2 p100∗

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Processing of NF-κB2 precursor protein p100 to generate p52 is tightly controlled, which is important for proper function of NF-κB. Accordingly, constitutive processing of p100, caused by the loss of its C-terminal processing inhibitory domain due to nfkβ2 gene rearrangements, is associated with the development of various lymphomas and leukemia. In contrast to the physiological processing of p100 triggered by NF-κB-inducing kinase (NIK) and its downstream kinase, IκB kinase α (IKKα), which requires the E3 ligase, β-transducin repeat-containing protein (β-TrCP), and occurs only in the cytoplasm, the constitutive processing of p100 is independent of β-TrCP but rather is regulated by the nuclear shuttling of p100. Here, we show that constitutive processing of p100 also requires IKKβ, but not IKKβ (IκB kinase β) or IKKY (IκB kinase γ). It seems that NIK is also dispensable for this pathogenic processing of p100. These results demonstrate a general role of IKKα in p100 processing under both physiological and pathogenic conditions. Additionally, we find that IKKα is not required for the nuclear translocation of p100. Thus, these results also indicate that p100 nuclear translocation is not sufficient for the constitutive processing of p100.

NF-κB represents a collection of dimeric transcription factors composed of members of the Rel family with five closely related DNA binding proteins: RelA (p65), RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52 (1–3). Whereas the three Rel proteins 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). Accordingly, the signaling leading to NF-κB activation can be classified into two major pathways, the canonical and non-canonical NF-κB pathways, which are based on inducible IκB degradation and p100 processing, respectively (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 β-TrCP ubiquitin ligase and degradation by the 26 S proteasome, thus allowing the NF-κB dimers to move to the nucleus to induce gene expression (10). Whereas IκB degradation predominantly depends on IKKβ and IKKγ, IKKα is largely dispensable for this event (10), although the nuclear function of IKKα is necessary for the transcription activity of NF-κB (12, 13).

In contrast, 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, 14, 15). Furthermore, under normal conditions, this novel NF-κB pathway only occurs in certain cell types at certain stages and responds to very limited stimuli (9), such as lymphotoxin β (LTβ) (9, 16), B-cell activating factor (BAFF) (17, 18), and CD40 ligand (19). These stimuli activate NIK, probably via up-regulation of NIK protein level by de novo protein synthesis and/or stabilization of NIK protein (20, 21). NIK then in turn activates IKKα and recruits it into p100 complex via the serine 866 and serine 870 of p100 (22). After recruited into the p100 complex, activated IKKα phosphorylates serines 99, 108, 115, 123, and 872 of p100 (22). The phosphorylation of these specific serines results in ubiquitination and subsequent processing of p100 mediated by the β-TrCP ubiquitin ligase and 26S proteasome, respectively (9, 20, 22–24).

Although p100 processing is hardly detected in most cell types including T cells, aberrantly persistent processing of p100 has been found in leukemic T cells transformed by the human T cell leukemia virus type I (HTLV-I), in which the processing of p100 is induced by the viral oncoprotein Tax (15). Like NIK, the physiological inducer, Tax also specifically targets IKKa to p100, triggering phosphorylation-dependent ubiquitination, and processing of p100 (15, 20, 24). Constitutive processing of p100 has also been found in various lymphomas associated with nfkβ2 gene rearrangements (15, 25). Interestingly, such genetic alterations always result in generation of C-termianlly truncated p100 proteins lacking the processing inhibitory domain (9, 25). Importantly, these C-terminal truncation mutants of p100 show oncogenic ability in vitro, and overexpression of p52 in the absence of p100 in p100 knock-in mice causes marked gastric and lymphocyte hyperplasia and early postnatal death of mice (26, 27). These findings strongly suggest that deregulated processing of p100 contributes to hu-

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The abbreviations used are: IKK, IκB kinase; NIK, NF-κB-inducing kinase; β-TrCP, β-transducin repeat-containing protein; HA, hemagglutinin; IB, immunoblotting; MEF, murine embryonic fibroblast; FITC, fluorescein isothiocyanate.

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nonspecific background band co-migrated with p52. Their processed product, p52 (p100 processing).

Recently, studies have suggested that the physiological and pathogenic processing of p100 is regulated by both common and different mechanisms (20). While NIK-mediated p100 processing depends on β-TrCP, constitutive processing of p100 is independent of this protein and is regulated by p100 nuclear shuttling (23, 24, 28). On the other hand, Tax-induced processing of p100 involves both mechanisms (20, 24). However, under all these conditions, the processing of p100 is generally regulated by a ternary domain of p100 consisting of both its N- and C-terminal sequences (20). Here, we provide both genetic and biochemical evidence demonstrating that the constitutive processing of p100 also requires IKKα but not IKKβ or IKKγ, suggesting another common mechanism for p100 processing. However, NIK, the activator of IKKα for inducible p100 processing under physiological conditions is not involved in constitutive processing of p100. We also find that IKKα and p100 phosphorylation are dispensable for p100 nuclear shuttling. It seems that p100 nuclear expression is not sufficient, though required, for constitutive processing, since the constitutive processing forms of p100 translocate into the nucleus of IKK null cells but defective in processing. Additionally, the phosphorylation-deficient mutants of the p100 constitutive processing forms fail to undergo processing, although they are still expressed in the nucleus.

MATERIALS AND METHODS

Expression Vectors and Antibodies—Expression vectors encoding p100 and its C-terminal deletion mutants (p100-(1–665) and p100-(1–454)), IKKα, HA-tagged NIK and its C-terminal mutant (NIK-c), have been described before (9, 20, 22). p100-(1–665) S/A and p100-(1–454) S/A constructs, which harbor serine to alanine substitutions at residues 99, 108, 115, and 123, were generated by site-directed mutagenesis as described (22, 24). The p100 mutants HuT-78 and LB-40, C-terminal HA-tagged p100-(1–665) and their S/A constructs were created by routine cloning strategy. The anti-IKKα antibody (anti-IKKα, H744) was bought from Santa Cruz Biotechnology. The anti-HA monoclonal antibody (anti-HA, 12CA5) was purchased from Roche Applied Science. The fluorescein isothiocyanate (FITC)-conjugated anti-mouse and anti-rabbit secondary antibody as well as Hoechst 33258 were from Amersham Biosciences and Molecular Probes, respectively. The antibodies used recognizing the N (anti-p100N) or C terminus (anti-p100C) of p100 were as described previously (9).

Cell Culture and Transfection—293 cells and murine embryonic fibroblasts (MEFs) derived from wild type, IKKα/−/−, IKKβ/−/−, IKKγ/−/−, and Aly/Aly mice were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. 293 and MEF cells were transfected with DEAE-dextran and FuGENE 6 reagent (Roche Applied Science), respectively (20, 29–31).

In Vivo p100 Processing Assays—293 or MEF cells were transfected by labeled expression vectors and lysed by radioimmunoprecipitation assay buffer (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) supplemented with a protease inhibitor mixture, followed by immunoblotting (IB) using p100N antibody as described previously (9, 14, 15).

Immunofluorescence Assays—MEF cells were transfected with p100, C-terminal HA-tagged p100-(1–665) (p100-(1–665)-HA) or its serine mutant, p100-(1–665) S/A-HA. After 24 h, the recipient cells were directly fixed, permeabilized, and sequentiy incubated with anti-p100C (for p100) or anti-HA (for p100-(1–665)-HA and its S/A mutant), followed by FITC-conjugated anti-rabbit or anti-mouse secondary antibodies. The subcellular localization of stained proteins was detected using an inverted fluorescence microscope. The cells were also counterstained with Hoechst 33258 for nuclear staining by detecting DNA as described previously (20).

RESULTS

The IKKα Phosphorylation Serines of p100 Are Essential for Its Constitutive Processing—Although we have demonstrated that IKKα is required for p100 processing induced by both NIK and Tax (14, 15), its role in the constitutive processing of p100 remains to be investigated. For this study, the N-terminal IKKα phosphorylation serines within p100, which play an essential role in inducible processing of p100 (22, 24), were mutated from four constitutive processing forms of p100: LB40, HuT-78, p100-(1–665), and p100-(1–454) (9, 20) (Fig. 1A). Among them, LB40 and HuT-78 proteins are expressed by the rearranged nfkβ gene in B cell chronic lymphocytic leukemia and T cell lymphoma, respectively (32, 33). The constitutive processing of these p100 constructs and their serine-alanine mutants was examined. Consistent with our previous studies (9, 20, 24), these p100 proteins exhibited constitutive processing (Fig. 1B, even lanes). Importantly, substitutions of the IKKα phosphorylation serines with alanines efficiently prevented their constitutive processing (Fig. 1B, odd lanes), suggesting a positive role of IKKα in constitutive processing of p100.

IKKα, but Not IKKβ or IKKγ, Is Required for Constitutive Processing of p100—To further confirm the significance of IKKα in the constitutive processing of p100, we expressed...
Eciently block NIK-induced p100 processing (Fig. 3). Consistent with our previous studies (15), NIK-c could efficiently block NIK-induced p100 processing (Fig. 3A, compare lane 3 with lane 2). However, NIK-c had no effect on constitutive processing of p100 (1–665) (compare lane 5 with lane 4). To further confirm this result, we also examined the processing of p100 (1–665), HuT-78, and LB40 in NIK Aly/Aly MEFs, which express mutated NIK protein and show defects in inducible p100 processing (9, 16). Consistent with the result of the blocking assays above, p100 (1–665) still underwent efficient processing in the NIK Aly/Aly MEFs (Fig. 3B, lane 6). These results strongly indicate that NIK is not required for constitutive processing of p100. Nevertheless, these results are consistent with the fact that the constitutive processing of p100 does not require external stimuli.

NIK Is Not Involved in Constitutive Processing of p100—We have recently shown that both IKKs and its upstream kinase NIK play an essential role in physiological processing of p100, although NIK is not required for Tax-induced p100 processing (9, 14, 15). It is thus very interesting and important to investigate the role of NIK in the constitutive processing of p100. Since the C-terminal portion of NIK (NIK-c) is known to function as a potent inhibitor of NIK (15, 29), p100 (1–665) together with an increasing amount of NIK-c was expressed in 293 cells. Consistent with our previous studies (15), NIK-c could efficiently block NIK-induced p100 processing (Fig. 3A, compare lane 3 with lane 2). However, NIK-c had no effect on constitutive processing of p100 (1–665) (compare lane 5 with lane 4). To
study suggested that the constitutive processing of p100 is regulated by p100 nuclear shuttling (28), although the role of IKKα in this event is still unknown. For this study, we performed immunofluorescence assays. In agreement with this previous study (28), p100 (1–665) was primarily located in the nucleus (Fig. 4, upper panel 2), although full-length p100 was only expressed in the cytoplasm (Fig. 4, upper panel 1). Disruption of IKKα phosphorylation sites did not affect the nuclear translocation of this p100 mutant (upper panel 3). Consistently, this p100 mutant was also able to move into the nucleus (Fig. 4, previous study (28), p100-(1–665) was primarily located in the nucleus, since the constitutive processing of p100 is weak and slow, compared with the inducible ubiquitination and processing of p100 (9, 23, 24). It appears that the unidentified ligase is located in the nucleus, since the β-TrCP-independent p100 processing is regulated by p100 nuclear shuttling, and β-TrCP-dependent processing only occurs in the cytoplasm (20). Clearly, efforts should be focused on identification of this ligase in the future.

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DISCUSSION

Constitutive processing of p100 due to the loss of its C-terminal processing-inhibitory domain contributes to tumorigenesis, it is therefore important to define the molecular mechanism regulating this pathogenic event. In this study, we have demonstrated that the constitutive processing of p100 is specifically regulated by IKKα and it-mediated phosphorylation of p100. We have also demonstrated that IKKβ/IKKγ and NIK, which are essential for canonical and non-canonical NF-κB signaling pathways, respectively, are not involved in this pathogenic event. Furthermore, we have shown that IKKα is dispensable for p100 nuclear translocation, further suggesting that the nuclear shuttling is not sufficient for its constitutive processing.

Although processing of p100 is usually tightly controlled, this proteolytic event could be efficiently activated at least by NIK, Tax, and the loss of the C-terminal portion of p100. While this proteolytic event could be efficiently activated at least by p100-(1–665) was primarily located in the nucleus, since the constitutive processing of p100 is weak and slow, compared with the inducible ubiquitination and processing of p100 (9, 23, 24). It appears that the unidentified ligase is located in the nucleus, since the β-TrCP-independent p100 processing is regulated by p100 nuclear shuttling, and β-TrCP-dependent processing only occurs in the cytoplasm (20). Clearly, efforts should be focused on identification of this ligase in the future.

ubiquitin ligase may be involved. In support of this idea, we have shown that ubiquitin ligase is located in the nucleus, since the β-TrCP-independent p100 processing is regulated by p100 nuclear shuttling, and β-TrCP-dependent processing only occurs in the cytoplasm (20). Clearly, efforts should be focused on identification of this ligase in the future.

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