Tax Deregulation of NF-κB2 p100 Processing Involves Both β-TrCP-dependent and -independent Mechanisms*

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Processing of the nf-kb2 gene product p100 to generate p52 is a tightly regulated event, consistent with the fact that the processing product, p52, is hardly detected in most cell types, including T cells, although the precursor p100 is expressed abundantly in these cells. However, in T cells transformed by the human T-cell leukemia virus type I (HTLV-I), p100 processing is very active, resulting in high level expression of p52. Because overproduction of p52 is associated with lymphoid hyperplasia and transformation, deregulation of p100 processing may be part of the oncogenic mechanism of HTLV-I. We demonstrated previously that HTLV-I Tax oncoprotein is a potent inducer of p100 processing through specific targeting of IKKα via IKKγ to p100 to trigger p100 phosphorylation and ubiquitination. In this study, we further show that Tax-mediated recruitment of IKKα to p100 requires serines 866 and 870 of p100, shown to be essential for inducible processing of p100. Upon interaction with p100, activated IKKα phosphorylates both N- and C-terminal serines of p100 (serines 99, 108, 115, 123 and 872), serving as a critical step in Tax-induced p100 processing. Using a genetic approach, we find that β-transducin repeat-containing protein, a component of the SCF ubiquitin ligase complex, previously shown to be required for physiological p100 processing mediated by nuclear factor-κB-inducing kinase, is only partially involved in Tax-induced processing of p100. These results indicate that both β-transducin repeat-containing protein-dependent and -independent mechanisms contribute to Tax-deregulated p100 processing, further suggesting the involvement of different mechanisms in cellular and viral pathways of p100 processing.

The oncogenic action of human T-cell leukemia virus type I (HTLV-I)† Tax protein involves activation of cAMP response element-binding protein/activating transcription factor proteins by directly interacting with their basic region-leucine zipper DNA-binding domains, thereby enhancing DNA binding (3–5). Unlike cAMP response element-binding protein/activating transcription factor, which is constitutively expressed in nucleus, NF-κB is normally sequestered in the cytoplasm by ankyrin repeat-containing inhibitors called IκB proteins (6). The nuclear expression of NF-κB can be induced by various cellular stimuli, such as T-cell mitogens, proinflammatory cytokines, and antigens. These stimuli trigger an IκB kinase (IKK) complex, which consists of IKKα, IKKβ (two catalytic subunits), and IKKγ (regulatory subunit, also named NEMO), to phosphorylate specific serines within the IκB sequence. The phosphorylated IκB is then targeted for ubiquitination and proteasome-mediated degradation, allowing the NF-κB to accumulate in the nucleus and transactivate target genes (7). Under normal conditions, this classic NF-κB activation is tightly controlled and usually transient (6, 7). However, in Tax-expressing cells, IKK is persistently activated, resulting in constitutive nuclear expression and transcription activity of NF-κB. Tax induces activation of IKK complex by directly interacting with the regulatory subunit IKKγ (8–12).

In mammalian cells, there are five NF-κB members: RelA (p65), RelB, c-Rel, p50, and p52, which function as various homo- and heterodimers (13). Unlike the Rel proteins, p50 and p52 are synthesized as large precursors NF-κB1 p105 and NF-κB2 p100, respectively (13–15). It is interesting that p105 and p100 also contain ankyrin repeats at their C-terminal regions and function as IκB-like inhibitors of NF-κB (16, 17). Different from the complete degradation of other IκB proteins, the proteasome-mediated degradation of p105 and p100 leads only to loss of their C-terminal ankyrin repeat regions, leaving intact N termini, p50, and p52, respectively (18, 19). Thus, the processing of p105 and p100 not only plays a role in liberating specific NF-κB complexes but also serves to generate p50 and p52. Although p105 processing is constitutive, the processing of p100 is tightly regulated through its inducible phosphorylation and polyubiquitination (20). Induction of p100 phosphorylation and subsequent processing is mediated by NF-κB-inducing kinase (NIK) and its downstream kinase IKKα (20, 21). It is interesting that neither IKKβ nor IKKγ is required for this novel, “non-canonical” NF-κB pathway, although both IKKβ and IKKγ are essential for canonical NF-κB activation (21, 22). Therefore, the NIK/IKKα-specific NF-κB pathway can not be stimulated by most of the classic NF-κB inducers; rather, it responds to signals involved in B cell differentiation and lymphoid organogenesis, including those triggered by lymphotoxin β (20, 23), B-cell activating factor (BAFF) (24, 25) and CD40 ligand (26). Those stimuli serve as physiological stimu-
lators of NIK to induce processing of p100.

Not surprisingly, proper processing of p100 plays an essential role in the development and maturation of lymphoid organs. For example, nfkβ2 gene-deficient mice with no expression of both p100 and p52 show severe defects in B cell function and impairment in the formation of the proper architecture of peripheral lymphoid organs (27, 28), a phenotype not observed in nfkβ1-deficient mice (29). Likewise, mice having other defects in this non-canonical NF-κB pathway, such as the lymphoxygen α or β receptor knockout mice (30, 31), the alaphympho- plasia (aly) mice (the NIK point mutant mice) (32), and the A/WysNj mice (the BAFF-receptor deletion mutant mice) (33), which have normal p100 expression but no p52 expression (20, 23–25), also show those phenotypes, albeit to different extents. On the other hand, mice whose nfkβ2 gene was engineered to contain a mutation that prevents expression of the p100 C terminus leading to constitutive p52 expression but no p100 production, develop lymphoid and gastric hyperplasia (34). In humans, developmental translocations that cause the rearranged nfkβ2 gene to lose its C-terminal processing inhibitory domain, leading to constitutive processing of p100 (20), are associated with the development of various lymphomas (35–37). In addition, those constitutively processed forms of p100 have been reported to oncogenically transform fibroblasts in vitro (38). It is interesting that deregulated p100 processing is also found to be associated with T-cell transformation by HTLV-I, an etiological agent of an acute and fatal T-cell maligancy, adult T cell leukemia (22). Because p100 processing is inefficient in T cells, including activated T cells (22), overproduction of p52 in T cells is a hallmark of HTLV-I infection and transformation (39). It is noteworthy that stable expression of p100 could efficiently block HTLV-I Tax-mediated transformation (40), through specific inhibition of the function of the alternative NF-κB pathway activated by HTLV-I (41). Thus, a fundamental understanding of p100 processing will not only provide important insights into the development of both human immune system and NF-κB-associated diseases but may also suggest effective therapeutic strategies for HTLV-I-induced adult T cell leukemia and other p100 processing associated diseases (42–44).

Our previous studies have demonstrated that Tax functions as a potent inducer of p100 processing (22). Unlike physiological p100 processing, which involves NIK and IKKα, but not IKKγ (20, 21), Tax induces p100 processing by specifically targeting IKKα indirectly via IKKγ into p100-containing complexes, triggering p100 ubiquitination and subsequent processing (22). We have recently discovered that NIK-mediated processing of p100 also involves recruitment of IKKα to p100. It is interesting that purified IKKα could phosphorylate serines located in both N- and C-terminal regions of p100, which are involved in NIK-mediated processing of p100 (45). In the present study, we have demonstrated that Tax recruitment of IKKα to p100 requires serines 866 and 870 of p100, which have been shown to be essential for inducible processing of p100. Tax-activated IKKα also consistently phosphorylates both N- and C-terminal serines of p100, a step required for the binding of p100 to β-TrCP E3 ligase and Tax-induced p100 processing. However, we also find that β-TrCP is only partially responsible for Tax-induced processing of p100, although NIK-mediated p100 processing depends on β-TrCP.

MATERIALS AND METHODS

Expression Vectors and Antibodies—Expression vectors encoding p100 and its serine mutants p100 S866A/S870A and p100 S866A/S870A/S872A (labeled p100 SS/AA and p100 SS/AAA, respectively), Tax, HA-tagged NIK, IKKα and its mutants, βTrCP and its siRNA-resistant form, and ubiquitin have been described previously (20, 22, 46, 47). Other p100 serine mutants were generated by site-directed mutagenesis using pCMV4 p100 as template with the use of the QuikChange site-directed mutagenesis kit (Stratagene). p100 S99A, p100 S108A, p100 S115A, p100 S123A, and p100 S872A harbor serine-to-alanine substitutions at residues 99, 108, 115, 123, and 872, respectively, p100 45SA and p100 55SA mutants were obtained by substituting four serines (Ser-99, -108, -115 and -123) or five serines (Ser-99, -108, -115, -123, and -872) with alanines, respectively (45). GST-p100C and GST-p100C SSS/AAA were generated previously (22). GST-p100 N-terminal mutant and its serine mutants were created by similar strategies. The anti-HA monoclonal antibody conjugated with horseradish peroxidase (anti-HA-HRP, 3F10) was purchased from Roche Molecular Biochemicals. The antibodies recognizing the N terminus of p100 (anti-p100N) and Tax were kindly provided by Dr. W. C. Greene (48, 49).

Cell Culture and Transfection—The kidney carcinoma cell line, 293, was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics. 293 cells (1 × 10⁶, seeded in a 6-well plate) were transfected with LipofectAMINE 2000 (Invitrogen) following the manufacturer’s instructions.

Immunoblotting (IB) and Co-Immunoprecipitation (Co-IP)—293 cells were transfected with the expression vectors as indicated in the figures and lysed 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, and 1 mM phenylmethylsulfonyl fluoride) supplemented with a protease inhibitor mixture, followed by IB or Co-IP assays as described previously (20, 22). The approximate amounts of cell lysates were 6 μg for IB and 300 μg for Co-IP assays.

Cell Labeling and Pulse-chase Assays—Transfected 293 cells were starved for 1 h in Dulbecco’s modified Eagle’s medium lacking methio- nine and cysteine, followed by metabolic labeling for 45 min with 350 μCi/ml [35S]methionine/[35S]cysteine. The pulse-labeled cells were chased for different times in complete Dulbecco’s modified Eagle’s medium supplemented with 10 μM unlabeled methionine and lysed in RIPA buffer. The radiolabeled, myc-tagged, wild-type p52 or its mutant was isolated by IP using anti-myc antibody, fractionated by SDS-PAGE, and visualized by autoradiography (20, 22).

In Vitro Kinase Assay—In vitro kinase assays were performed as described previously (11, 12). Cell extracts from HTLV-I-transformed cells were prepared and subjected to immunoblotting or to conformation assays.

In Vivo Ubiquitin Conjugation Assays—293 cells were transfected with HA-tagged ubiquitin and p100 or its mutants in the presence or absence of Tax. 36–48 h after transfection, the cells were lysed in RIPA buffer and subjected to immunoprecipitation (IP) using anti-p100. Agaro- beads were washed three times with RIPA buffer followed by two washes with RIPA buffer supplemented with 1% urea. The ubiquitin-conjugated p100 eluted by SDS loading buffer was analyzed by IB using anti-HA-HRP (20, 22).

siRNA-mediated Gene Silencing—The sequences of double-stranded siRNA are: for β-TrCP, GUG GAA UUU GUG GAA CAU CTT (sense) and GAU GUU CCA AUU GUG GAA CAU CT (antisense); and for green fluorescein protein, GUU ACC UGU UGC GCC ATT (sense) and UGG CCA UGG AAC AGG UAG CTT (antisense). These siRNAs were synthesized by Dharmacon Research, Inc. (Lafayette, CO).

293 cells were transfected using LipofectAMINE 2000 (Invitrogen) in accordance with the manufacturer’s instructions. In brief, 293 cells were transfected in 6-well plates with 60 pmol of siRNA mixed with 800 ng of carrier DNA (pCMV4 vector). Transfection was repeated 24 h later to achieve high efficiency of siRNA transfection, except that the indicated DNA expression vectors were used in place of carrier DNA. Twenty-four hours after the second transfection, whole-cell extracts were prepared and subjected to immunoblotting or to in vivo ubiquitin conjugation assays.

RESULTS

Tax Recruitment of IKKα into p100 Complexes Is Independent of the Kinase Activity of IKKα—As we reported previously, Tax could specifically target IKKα, but not IKKβ, into p100 complexes (22). To further test whether the kinase activity of IKKα or its active status is required for p100/IKKα association, both kinase constitutively active (IKKα SS/EE) and inactive (IKKα SS/AA or IKKα KA) forms of IKKα were used. As shown...
Tax actually slightly reduced the binding between IKKα and these two mutants of p100 (top, compare lanes 9 and 11 with lanes 10 and 12, respectively). The decreased interaction could be caused by the competition of the p100 mutants and Tax for IKKα. These results indicated that the N-terminal sequences of p100 are not involved in Tax-induced IKKα binding of p100, but rather inhibit the constitutive activity of p100 in binding to IKKα. These studies further suggested that the IKKα binding site of p100 is located in its C terminus.

Although p100 Δ1–32 and p100 Δ1–71 still responded to Tax-induced p100 processing (Fig. 2B, bottom, lanes 4 and 6), the p100 Δ1–124 mutant was largely resistant to Tax-induced processing (lane 8), although it still retained the ability to bind to Tax and to recruit IKKα mediated by Tax (top and second panel, lane 8). It is noteworthy that Tax could still induce marginal processing of the p100 Δ1–124 mutant (Fig. 2C, lane 4), suggesting that other sequences besides amino acid 1–124 of p100 might contribute to this event. Nevertheless, these results suggest that the sequences between residue 71 and 124 play an important role in Tax-mediated p100 processing. In contrast, p100 Δ1–183 and p100 Δ1–343 were not processed when Tax was co-expressed, but instead were degraded, as indicated by the loss of the precursors without appearance of processing products (Fig. 2B, bottom, lanes 10 and 12; Fig. 2C, lane 6). The failure to detect processing products was not a result of their instability, as the p52 Δ1–183 (the presumptive processing product of p100 Δ1–183) showed a half-life similar to that of p52 in our pulse-chase assays (Fig. 2D). The degradation of p100 Δ1–183 and p100 Δ1–343 induced by Tax seemed to be mediated by endogenous IKK, because Tax could not bind to these two p100 mutants (Fig. 2B, second panel, lanes 10 and 12). IKKα or IKKβ consistently induced even more dramatic degradation of p100 Δ1–183 (Fig. 2E). These results indicated that once it has lost the N-terminal sequences essential for inducible processing, p100 behaves similarly to typical IκB proteins in undergoing complete degradation.

**Tax-mediated Recruitment of IKKα into p100 Complexes Requires Serines 866 and 870 of p100**—Consistent with the results that the N-terminal sequences of p100 are not required for Tax-mediated recruitment of IKKα into p100 complexes, loss of 41 or more amino acids from the C terminus of p100 prevented Tax-mediated IKKα recruitment (Fig. 3B, top, lanes 4–9), although these p100 mutants still retained the ability of wild-type p100 to bind to Tax (second panel from top, lanes 3–9). These results further supported our previous studies showing that Tax associates with p100 via two N-terminal α-helices of p100 (22), and indicated that the last 41 amino acids are required for Tax-mediated IKKα recruitment to p100.

Because serines 866 and 870 located in this region have been shown to be essential for inducible processing of p100 (20, 22), we tested the IKKα binding activity of the p100 S866A/S870A mutant harboring these two serine-to-alanine substitutions, in the presence of Tax expression. As a control, the p100 S872A mutant, which harbors substitution of serine 872 with alanine, was also included in these experiments. It is interesting that mutation of serines 866 and 870, but not serine 872, blocked the recruitment of IKKα in Tax-expressing cells (Fig. 3C, top, lanes 4 and 6, respectively). The failure of the p100 S866A/S870A mutant to recruit IKKα was not caused by its inability to bind to Tax, because, like wild-type p100 and the p100 S872A mutant, p100 S866A/S870A could efficiently bind to Tax (second panel from top, compare lane 4 with lanes 2 and 6). In fact, mutation of all three of these serines did not affect p100/ Tax interaction (second panel, lane 8). Consistent with the significance of serines 866 and 870 in IKKα recruitment, the three-serine mutant of p100 (p100 S866A/S870A/S872A) also
failed to interact with IKKs when Tax was coexpressed (top, lane 8). Parallel immunoblotting assays revealed that the expression levels of all the proteins were comparable (third and bottoms). It is noteworthy that the p100 mutants 1–753, 1–665, and 1–454 are actually constitutive processing forms (Fig. 3B, bottom, lanes 5–7), because these mutants also lose processing inhibitory domains (20, 50). The mechanism of constitutive processing of p100 is different from that of inducible processing and seems to be independent of both IKK/H9251 and IKK/H9252–TrCP (47, 50).

Nevertheless, these data clearly indicated that serines 866 and 870 are essential for Tax-mediated recruitment of IKK/H9251 to p100 and subsequent processing of p100. Serine 872 of p100, an IKK/H9251 Phosphorylation Site, Contrib-utes to Tax-induced Processing of p100—Although it is not involved in binding to Tax or in Tax-mediated p100/IKK/H9251 interaction, the N terminus of p100 contains sequences required for Tax-induced p100 processing but dispensable for Tax-mediated p100/IKK/H9251 interaction.

**FIG. 2.** The N terminus of p100 contains sequences required for Tax-induced p100 processing but dispensable for Tax-mediated p100/IKK/H9251 interaction. A, schematic representation of p100 and its truncation mutants. The α-helices, the glycine-rich region (GRR), the processing site, the ankyrin repeat domain (ARD), and the C-terminal processing-required sequences (C-PRS) are indicated. p52 is equivalent to p100 (1–405). B, the N-terminal sequences are not required for Tax-mediated recruitment of IKK/H9251 into p100 but are essential for Tax-induced p100 processing. HA-tagged IKK/H9251 and myc-tagged p100 or its N-terminal deletion mutants were transfected into 293 cells either in the absence (+) or presence (−) of Tax, followed by IP using anti-myc antibody. The co-precipitated IKK/H9251 and Tax (second panel from top) were detected by immunoblotting using anti-HA-HRP and anti-Tax, respectively. The expression levels of HA-tagged IKK/H9251 (third panel) and Tax (fourth panel), as well as the p100 constructs and their processing products (bottom), were monitored by direct IB using cell lysates. Note, myc-tagged p52 (processing products of myc-p100) co-migrated with a background band labeled with an asterisk in the bottom panel. C, the first 124 N-terminal amino acids of p100 are essential for Tax-induced p100 processing, whereas further deletion leads to inducible degradation of p100. Five times more protein extracts than used in B were loaded for SDS-PAGE, followed by IB using higher concentration of anti-myc to detect low processing of p100 N-terminal deletion mutants. Again, a background band existed in all the lanes, indicated as NS. D, p52 and p52Δ1–183 show similar stabilities. 293 cells were transfected with p52 or its N-terminal deletion mutant p52Δ1–183. The cells were pulse-labeled for 45 min followed by chase for the indicated times. p52 and its mutant p52Δ1–183 are indicated. E, p100Δ1–183 mutant behaves like an IκB, exhibiting degradation induced by IKK/H9251, IKK/H9252, or Tax. The p100Δ1–183 mutant was transfected into 293 cells with an empty vector or the indicated cDNA, followed by IB assays using the indicated antibodies.
Tax-induced processing (Fig. 3C, bottom, compare lane 6 with lane 2), a result observed in multiple experiments (Fig. 4B and data not shown). Thus, the three serines within the p100 C terminus are critical for Tax-induced interaction of p100/IKKα. 293 cells were transfected with HA-tagged IKKα and p100 or its C-terminal deletion mutants either in the absence (−) or presence (+) of Tax. Cell lysates were subjected to IP using anti-p100N for examining the p100/IKKα interactions or to IB using the indicated antibodies for detection of the expression levels of IKKα, p100 and its mutants as well as their processing products p52 (same as p100 (1–405)). Note that p100 (1–859) loses processing inducible domain, whereas other p100 C-terminal truncation mutants (1–753, 1–665, and 1–454) undergo constitutive processing caused by loss of the processing inhibitory domain (see text and Refs. 20, 47, and 50 for details). These p100 C-terminal deletion mutants have been described in Fig. 2A. C, serines 866 and 870 but not serine 872 of p100 are essential for Tax-induced interaction of p100/IKKα. 293 cells were transfected with indicated expression vectors, followed by immunoprecipitation and immunoblotting assays as described in A. The ratio of p52 to p100 is indicated in the figure. S/AA, SSS/AA, and SSS/AAA represent the p100 S866A/S870A, p100 S866A/S870A/S872A point mutants, respectively. D, serine 872, but not serine 866 or serine 870, of p100 is an IKKα phosphorylation site. Tax-activated IKKα was isolated from the HTLV-I-transformed T-cell line, C8166, by IP using anti-IKKα and subjected to in vitro kinase assays using the indicated substrates: lane 1, GST-p100 (860–900) wild type; lane 2, GST-p100 (860–900) mutant harboring substitutions of serines 866, 870, and 872 to alanines; lane 3, GST-p100 (860–900) mutant harboring substitutions of serines 866 and 870 to alanine; lane 4, GST-p100 (860–900) mutant harboring serine 872 to alanine substitution (indicated as WT, SSS/AA, SSS/AA, and S/AAA at the bottom of the figure, respectively). The phosphorylated GST-p100 (860–900) and its serine-to-alanine substitution mutants are indicated as P-p100C (top), and the levels of the wild-type and mutant forms of p100 substrates as well as IKKα were also monitored by IB analysis of the kinase assay membrane using anti-GST (middle) and anti-IKKα (bottom).

Serine 872 of p100 is involved in Tax-induced p100 processing, but not Tax-mediated recruitment of IKKα to p100, which suggests that this serine might be involved in other events mediated by Tax. One possibility is that Tax-targeted IKKα may phosphorylate serine 872. In this regard, our previous studies have indicated that serines 866, 870, and 872 of p100 are candidate sites for IKKα phosphorylation (22). More recently, we have found that purified IKKα protein phosphorylates serine 872, but not serines 866 and 870, of p100 (45), although it remains unknown whether Tax-activated endogenous IKKα in HTLV-I-transformed T cells also induces this phosphorylation. To further confirm that, in vitro kinase assays were performed using glutathione S-transferase (GST)/p100 C-terminal fusion proteins (GST-p100 (860–900)) harboring mutations of serines 866 and 870 or serine 872, converting them to alanine residues. As expected, GST-p100 (860–900) S866A/S870A mutant could be phosphorylated equivalently to wild-type GST-p100 (860–900) by IKKα from HTLV-I-transformed T cells (Fig. 3D, top, compare lane 3 with lane 1), whereas mutation of serine 872 completely blocked the C-terminal phosphorylation of p100 by IKKα (lane 4). Thus, serine 872, but not serine 866 or 870, is an actual target of Tax-activated IKKα, even though serines 866 and 870 are required for the recruitment of IKKα into p100 complexes and subsequent processing of p100 induced by Tax.
For example, S99A harbors a substitution of serine 99 by alanine. 4S/A harbors substitutions of serines 99, 108, 115, and 123, whereas 5S/A harbors substitutions of p100 and p52 (p100 processing) (*bottom*), and Tax (*top*). The new p100 mutants were designated based on the position of the mutated serine.

C, serines 99, 108, 115, and 123 are targets of Tax-activated IKK. Suggested that there may be additional IKK not involved in Tax-mediated recruitment of IKK as well as immunopurified IKK. IKK was further substantiated by our recent discovery showing that IKK123 of p100. 293 cells were transfected with the indicated p100 constructs either in the absence (−) or presence (+) of Tax, followed by IB analysis of p100 and p52 (p100 processing) (*top*), and Tax (*bottom*). The new p100 mutants were designated based on the position of the mutated serine.

To further confirm that these N-terminal serines are also targets of Tax-activated IKKa from HTLV-I-transformed T cells, GST fusion proteins containing the N-terminal 132 amino acids of p100 (GST-p100 (1–132)) were used for *in vitro* kinase assays. Phosphorylated GST fusion mutants are shown at the *top*; levels of the GST fusion proteins are shown at the *bottom*. The GST fusion proteins were named using the same strategy as described in *B*. D. IKKa phosphorylation sites are dispensable for Tax-induced p100 binding to IKKa. The indicated p100 constructs and HA-tagged IKKa were transfected alone (−) or together with Tax (+), followed by Co-IP assays using anti-p100N. The co-precipitated IKKa (*top*) and Tax (*second panel from top*) as well as the expression levels of IKKa (*third panel*), p100, and p52 (*bottom*) are shown in the figure.

**Fig. 4.** N-terminal IKKa-targeted serines 99, 108, 115, and 123 of p100 play important roles in Tax-induced p100 processing but are not involved in Tax-mediated recruitment of IKKa. A, schematic representation of p100 showing both the N- and C-terminal IKKa phosphorylation sites. The processing site is also indicated. B, Tax-induced processing of p100 involves the N-terminal serines 99, 108, 115, and 123 of p100. 293 cells were transfected with the indicated p100 constructs either in the absence (−) or presence (+) of Tax, followed by IB analysis of p100 and p52 (p100 processing) (*top*), and Tax (*bottom*). The new p100 mutants were designated based on the position of the mutated serine. For example, S99A harbors substitutions of serine 99 by alanine. 4S/A harbors substitutions of serines 99, 108, 115, and 123, whereas 5S/A harbors substitutions of serines 99, 108, 115, 123, and 872. SSI/AA is the p100 S866A/A870A mutant. The ratio of p52 to p100 is indicated in the figure. C, serines 99, 108, 115, and 123 are targets of Tax-activated IKKa. GST-p100 (1–132) wild-type and its mutants harboring serine substitution(s), as well as immunopurified IKKa from HTLV-I-transformed T cells, were used for *in vitro* kinase assays. Phosphorylated GST fusion mutants are shown at the *top*; levels of the GST fusion proteins are shown at the *bottom*. The GST fusion proteins were named using the same strategy as described in *B*. D, IKKa phosphorylation sites are dispensable for Tax-induced p100 binding to IKKa. The indicated p100 constructs and HA-tagged IKKa were transfected alone (−) or together with Tax (+), followed by Co-IP assays using anti-p100N. The co-precipitated IKKa (*top*) and Tax (*second panel from top*) as well as the expression levels of IKKa (*third panel*), p100, and p52 (*bottom*) are shown in the figure.

**IKKα Phosphorylation Sites in the N Terminus of p100 Are Important for Tax-induced p100 Processing**—The finding that the C-terminal IKKα phosphorylation serine 872 contributes to but is not sufficient for Tax-induced p100 processing (Fig. 3) suggested that there may be additional IKKα targets within p100 that are also involved in p100 processing. Because the sequences between amino acid residues 71 and 124 play a crucial role in Tax-induced p100 processing (Fig. 2B), we hypothesized that serines (serines 99, 108, 115, and 123) located in this region might also be targets of IKKα. This hypothesis was further substantiated by our recent discovery showing that IKKα can also phosphorylate these serines in *vitro* (45). At first, the functional significance of these serines in Tax-induced p100 processing was investigated by using p100 mutants harboring individual or combinational serine substitutions in *in vivo* inducible processing assays. As shown in Fig. 4B, all the single-serine substitution mutants, such as the p100 S872A mutant, showed appreciable defects in Tax-induced processing, although to different extents. These defects were much clearer when all the four serines (p100 4S/A) were simultaneously substituted (*lane 14*), although mutation of these four serines together with serine 872 (p100 5S/A) completely blocked Tax-induced p100 processing (*lane 16*). Thus, both N- and C-terminal serines within p100 play important roles in Tax-induced processing of p100.

**IKKα and β-TrCP in Tax-mediated p100 Processing**—Because serines 866 and 870 of p100 are required for Tax-induced IKKα recruitment to p100, it was interesting to test whether other IKKα phosphorylation serines within p100 are also involved in Tax-mediated p100/IKKα association, even though serine 872 is not involved (Fig. 3C). Like the p100 S872A mutant, the IKKα phosphorylation-deficient mutant of p100 (p100 5S/A), which harbors substitutions of the five IKKα phosphorylation serines by alamines, remained competent in interacting with IKKα in Tax-expressing cells (Fig. 4D, *top*, *lane 4*). These findings suggested that the recruitment of IKKα to p100 by Tax and IKKα-mediated phosphorylation...
lysates were subjected to IP using anti-p100, and the co-precipitated proteins were analyzed by IB using anti-HA-HRP to detect the ubiquitin-conjugated p100 as described in \( \text{Fig. 5A} \). To further confirm the functional significance of these serines in the recruitment of \( \beta\)-TrCP, we tested the possible physical interaction between the E3 ligase, \( \beta\)-TrCP, and p100 in Tax-expressing cells by Co-IP assays. As shown in Fig. 5A, in the absence of Tax, \( \beta\)-TrCP only weakly bound to p100 (top, lane 1). However, a significant interaction between \( \beta\)-TrCP and p100 was demonstrated when Tax was co-expressed (lane 2). To examine the role of IKK\( \alpha \) phosphorylation sites within p100 in \( \beta\)-TrCP/p100 interactions induced by Tax, we also included the IKK\( \alpha \) phosphorylation-deficient mutant of p100, p100 SS/A, in the Co-IP assays. Consistent with its resistance to Tax-induced processing, this p100 mutant failed to interact with \( \beta\)-TrCP either in the absence or presence of Tax (top, lanes 3 and 4). To further confirm the functional significance of these serines in the recruitment of \( \beta\)-TrCP, in vivo ubiquitin conjugation assays were also performed using this p100 mutant. As expected, the p100 SS/A mutant was indeed unable to become polyubiquitinated in Tax-expressing cells, although Tax could efficiently induce ubiquitination of wild-type p100, as evidenced by the formation of an ubiquitin-conjugated heterogeneous ladder (Fig. 5B, top, lanes 4 and 5, respectively). In agreement with its inability to bind to IKK\( \alpha \) and be phosphorylated, p100 SS66A/SS70A also failed to bind to \( \beta\)-TrCP and to become ubiquitinated in Tax-expressing cells (Fig. 5B, A and B, top, lane 6). These results demonstrated the significance of the IKK\( \alpha \)-targeted serines of p100 in the recruitment of the E3 ligase, \( \beta\)-TrCP, into p100 as part of Tax-induced p100 processing. These results also suggested that
the potential role of β-TrCP in Tax-induced p100 processing is to mediate ubiquitination of p100.

**β-TrCP Contributes to Tax-induced Polyubiquitination and Processing of p100**—As we have shown above and before, Tax-induced p100 processing is associated with the polyubiquitination of p100 (22; see also Fig. 5B). To confirm the significance of β-TrCP in Tax-mediated p100 ubiquitination and processing, the siRNA-mediated gene suppression technique was used. As shown by IB, transfection of β-TrCP siRNA (Fig. 5, C and D, even-numbered lanes) efficiently depleted the expression of β-TrCP but not that of other proteins, such as p100, Tax, and NIK, whereas the control green fluorescent protein siRNA (odd-numbered lanes) did not affect the expression of these proteins (Fig. 5C, third panel from top, compare lane 11 with lane 12 for suppression of β-TrCP; top, lanes 1–12 for p100; bottom, lanes 3–6 for Tax; and second panel, lanes 8–10 for NIK). A β-TrCP mutant (β-TrCP[K476R]) harboring sense mutations in the siRNA targeting site but not affecting the protein sequence showed resistance to β-TrCP siRNA (third panel, lanes 5–8). It is noteworthy that Tax-induced processing of p100 was appreciably, although not completely, abrogated in β-TrCP-deficient cells (top, lane 4), whereas transfection of β-TrCP[R476K] could efficiently restore this ability of Tax (lane 6). Suppression of β-TrCP also dramatically and consistently blocked Tax-induced p100 ubiquitination (Fig. 5D, lane 4), and this defect could also be reverted by transfection of β-TrCP[R476K] (lane 6). In agreement with a previous study (47), suppression of β-TrCP prevented NIK-mediated ubiquitination and processing of p100 (Fig. 5, C and D, lane 10). It is noteworthy that Tax, but not NIK, could still induce moderate processing of p100 in β-TrCP-deficient cells (Fig. 5C, top, compare lane 4 with lane 10), suggesting that a β-TrCP-independent mechanism also contributes to Tax-induced processing of p100. Nevertheless, these results indicated that β-TrCP is indeed an E3 ligase important for both ubiquitination and processing of p100 induced by Tax.

**DISCUSSION**

In this study, we have explored the detailed mechanisms by which the HTLV-I-encoded oncoprotein, Tax, deregulates the tightly controlled processing of p100 for its oncogenic action. We demonstrated that both N- and C-terminal serines of p100 are involved in Tax-induced p100 processing. Consistent with our previous studies showing their functional significance in inducible p100 processing (20, 22), the data here further indicate that serines 866 and 870 of p100 are actually required for Tax-mediated IKKα recruitment into p100 complexes. Besides the C-terminal serine 872, Tax-activated IKKα also phosphorylates four N-terminal serines (residues 99, 108, 115, and 123) of p100, which play a critical role in Tax-induced p100 processing. We also showed that β-TrCP, which is required for NIK-mediated ubiquitination and processing of p100, is just partially involved in Tax-mediated p100 processing. We have demonstrated recently that Tax-, but not NIK-, mediated p100 processing requires IKKα (22). The data presented here thus provide a second body of evidence for the involvement of different mechanisms in cellular and viral pathways of p100 processing. These findings provide the molecular basis for a possible approach to HTLV-I diseases by specifically blocking Tax-deregulated p100 processing.

Unlike p105 processing, which is largely constitutive, p100 processing is highly selective and tightly controlled (7, 19, 44). Therefore, in most cell types, including T cells, only very small amounts of p52 are produced relative to the levels of its precursor p100 (20, 22). However, in HTLV-I-transformed T cells, p100 is actively processed, resulting in p52 overproduction (22, 39). Because overproduction of p52 is associated with lymphoid hyperplasia and transformation (42, 44), deciphering the mechanisms of Tax-deregulated processing of p100 will help understand the development of HTLV-I-mediated adult T cell leukemia and other NF-κB-associated diseases. Like IκB degradation in the canonical NF-κB pathway, Tax also usurps the IKK complex to induce processing of p100 (22). However, it seems that whereas both IKKα and IKKβ are involved in Tax activation of classic IκB degradation (51, 52), Tax specifically targets IKKα into p100 complexes to initiate processing of p100 (22). IKKγ, an essential adaptor for binding of Tax to IKKα and/or IKKβ (8–12), is required for both Tax-activated canonical and non-canonical NF-κB pathways.

Our current study shows that Tax recruitment of IKKα to p100 requires serines 866 and 870 of p100, two serines essential for inducible processing of p100 (Fig. 3). It seems that recruitment of IKKα to p100 and phosphorylation of p100 by IKKα are two sequential steps in Tax-induced p100 processing, because p100 S866A/S870A defective in IKKα binding cannot be phosphorylated (Fig. 3D; Ref. 22), whereas a p100 phosphorylation-deficient mutant is still capable of binding to IKKα in Tax-expressing cells (Fig. 4). The function of p100 phosphorylation would then act to recruit the β-TrCP SCF complex, which mediates polyubiquitination and subsequent processing of p100 (Fig. 5). Our genetic and biochemical studies also suggest another novel function of p100 phosphorylation in inducible processing of p100. This new function seems to be β-TrCP-independent, because the recruitment of IKKα to p100 and subsequent phosphorylation of p100 are completely required for Tax-induced p100 processing, whereas in β-TrCP-deficient cells, Tax-induced processing of p100 is only partially inhibited (Fig. 5C). It seems that the β-TrCP-independent mechanism of inducible processing of p100 is Tax-specific. Unlike Tax-deregulated processing of p100, p100 processing induced by NIK, the physiological stimulator of p100 processing, is completely blocked in β-TrCP-deficient cells (Fig. 5; Ref. 47). Although the mechanism of the β-TrCP-independent pathway for p100 processing is currently unclear, a previous study indicated that the processing of the constitutive forms of p100 is also through a β-TrCP-independent mechanism (47). It is noteworthy that the constitutive processing of p100 is much slower than that of inducible p100 processing mediated by NIK, whereas the dynamics of Tax-mediated p100 processing is intermediate (data not shown; Refs. 20, 22), further suggesting that both β-TrCP-dependent (rapid processing) and -independent (slow processing) mechanisms are involved in Tax-mediated processing of p100.

Although different mechanisms may exist for HTLV-I-mediated induction of the non-canonical NF-κB pathway, the current study also provides some insights into the tightly controlled, physiological processing of p100. Our data demonstrate that serines 866 and 870 of p100, which have previously been shown to be essential for both NIK- and Tax-induced p100 processing (20, 22), are required for the binding of p100 to IKKα, a key step for inducible p100 processing. These results further confirm that physical recruitment of IKKα to p100 and subsequent phosphorylation of p100 by IKKα are the general mechanisms of inducible processing of p100, which is also consistent with our recent discoveries indicating that IKKα is required for inducible processing of p100 under both physiological and pathogenic conditions (21, 22). Indeed, both the N- and C-terminal IKKα phosphorylation sites of p100, similar to IKKα docking site in the C terminus, play an essential role in p100 processing induced by NIK or Tax. Mutation of individual IKKα phosphorylation serines causes a partial block in Tax or NIK-induced p100 processing, whereas substitution of all of them completely prevents the processing of p100 induced by Tax or NIK (Figs. 3 and 4; Ref. 45).
There is an intriguing question of why the multiple IKKα-targeted serines in p100, unlike the IKK targeted serines within other proteins, such as IxBα, are located at distant positions in the amino acid sequence of the protein. Because the Rel homology domain of NF-κB proteins can strongly associate with the ankyrin repeat domain of IxBα, it seems plausible that p100 N-terminal Rel homology domain interacts with its C-terminal ankyrin repeat domain, thereby bringing the C-terminal serines close to the N-terminal serines to form a functional domain in three dimensions. One other important function of the interaction between the N and C termini of p100 seems to be to hide the access of p100 to IKKα, because IKKα fails to directly associate with p100 (Fig. 1). In support of this hypothesis, the deletion of the N-terminal 183 amino acids or more results in constitutive IKK binding of p100 (Fig. 2). It is interesting that in Tax-expressing cells, IKKα can stably associate with p100 (Figs. 1–4; Ref. 22). It is obvious that the “adaptor” function of Tax contributes to the recruitment of IKKα to p100. Most probably, the interaction between Tax and p100 also changes this three-dimensional domain to facilitate exposure of the C-terminal “docking” site of p100 (serines 866 and 870). It is noteworthy that two N-terminal α-helices of p100 required for Tax binding to p100 are located near the N-terminal phosphorylation sites of IKKα, suggesting these two helices may be part of this functional three-dimensional domain. Further supporting this idea, our data also indicate that loss of the p100 N-terminal region, including the two helices and N-terminal phosphorylation serines, leads to inducible degradation instead of processing of p100 (Fig. 2). This hypothesis can further explain why signal-dependent phosphorylation of p105, a protein very homologous to p100, mainly triggers degradation but not processing, because positions of the two α-helices are the biggest structural difference between these two proteins at their N termini (53). This idea clearly needs to be further substantiated by more studies, particularly structural studies of full-length p100 and p105.

Based on our findings, a model of Tax-induced processing of p100 is presented in Fig. 6. In brief, in HTLV-I-infected and Tax-expressing cells, Tax directly interacts with p100 through its two N-terminal α-helices, probably changing the conformation of p100 to expose the two C-terminal “docking” serines 866 and 870. At the same time, Tax activates IKKα indirectly via interacting with IKKγ. Then Tax-activated IKKα binds to the exposed serines 866 and 870 of p100 with the additional help (an adaptor function) of Tax. Once stably associated with p100, IKKα phosphorylates both N- and C-terminal serines within p100, which triggers the interaction between p100 and β-TrCP and subsequent β-TrCP-mediated p100 ubiquitination and processing of p100. A β-TrCP-independent mechanism may also contribute to Tax-deregulated processing of p100, although the detailed mechanism remains to be elucidated.

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