Proteasome-mediated processing of the nfb2 gene product p100 is a regulated event that generates the NF-xB subunit p52. This event can be induced through p100 phosphorylation by a signaling pathway involving the nuclear factor-xB-inducing kinase (NIK). The C-terminal region of p100, which contains its phosphorylation site and a death domain, plays a pivotal role in regulating the processing of p100. To understand the biochemical mechanism of p100 processing, we searched for cellular factors interacting with the C-terminal regulatory region of p100 using the yeast two-hybrid system. This led to the identification of S9, a non-ATPase subunit of the 19 S proteasome with no known functions. Interestingly, the S9/p100 interaction could be induced by NIK but not by a catalytically inactive NIK mutant. This inducible molecular interaction required p100 ubiquitination and was dependent on the intact death domain. We further demonstrated that the death domain is essential for NIK-induced post-translational processing of p100, thus providing a functional link between the S9 binding and the processing of p100. Finally, we provide genetic evidence for the essential role of S9 in the inducible processing of p100.

The NF-xB family of transcription factors plays a pivotal role in diverse biological processes, including innate and adaptive immune responses, cell growth, differentiation, and survival (1–3). In mammalian cells, this family is composed of five members, RelA, RelB, c-Rel, p50, and p52, that function as homo- and heterodimers (4). The NF-xB dimers are normally sequestered in the cytoplasmic compartment as inactive complexes by specific inhibitory molecules that belong to the inhibitor of NF-xB (IxB) family. Activation of NF-xB by cytokines and various other cellular stimuli involves phosphorylation and proteolysis of the IxB proteins and the concomitant nuclear translocation of NF-xB factors. This acute activation process is mediated by an IxB kinase (IKK), which is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (5). Upon activation by upstream signals, IKK phosphorylates the IxBs at specific serines, which targets these inhibitors for ubiquitination and proteasome-mediated degradation. In addition to this so-called canonical pathway of activation, NF-xB is subjected to regulation by other mechanisms. These include processing of the NF-xB1 and NF-xB2 precursor proteins p105 and p100, which is required for generating the p50 and p52 NF-xB subunits, respectively (4, 6). Because p105 and p100 function as IxB-like molecules, their processing also leads to activation of specific NF-xB members.

Recent studies suggest that the processing of p105 and p100 involves different mechanisms (3, 7). Whereas the processing of p105 is constitutive, the processing of p100 is tightly controlled (8). The lack of constitutive processing of p100 is largely attributable to its C-terminal processing-inhibitory domain, which contains a region known as a death domain (DD). Active processing of p100 can be induced by the NF-xB inducing kinase (NIK) (8), which seems to function through its downstream kinase IKKα (9). Once phosphorylated, p100 is recognized by the SCF(xC)-ubiquitin ligase, which catalyzes its polyubiquitination (10). This modification serves as an essential trigger for the inducible processing of p100 by the proteasome.

The 26 S proteasome complex is composed of two subcomplexes, the 20 S catalytic particle and the 19 S regulatory particle (11). The 20 S particle has a cylinder-shaped structure containing four stacked heptameric rings that form the proteolytic chamber. The 20 S cylinder is capped on both sides by a 19 S particle to form the 26 S proteasome. The 19 S particle is composed of at least 17 proteins that form two subcomplexes; the “base” complex is composed of six AAA family of ATPases and three other proteins, and the “lid” complex is composed of eight non-ATPase proteins (12). It is generally believed that the 19 S particle plays two major roles in facilitating protein degradation by the 20 S particle. The first is to recognize and position ubiquitinated protein substrates, and the second is to unfold the protein substrates so that they can be inserted into the proteolytic chamber of the 20 S catalytic particle. How these events occur in the 19 S particle is unclear, and how the individual subunits participate in the regulatory functions also remains unknown.

In the present study, we demonstrated that S9, a protein located in the “lid” of the 19 S proteasome (13), specifically interacts with p100. The S9/p100 interaction could be strongly induced by NIK and required p100 ubiquitination. Interestingly, the DD of p100 was essential for S9 binding as well as for NIK-induced post-translational processing of p100. These results provide an important insight into the biochemical mechanisms of p100 processing in the proteasome.
Yeast Two-hybrid Screening—Yeast two-hybrid screening was performed using the MATCHERLEXA two-hybrid system (Clontech). A cDNA fragment encoding the C-terminal region (amino acids 754–900) of p100 was cloned into the pLEXA vector downstream of the DNA-binding domain of LexA to generate the pLEXA-p100C bait. Initial tests showed that this bait has no self-transactivation activity (data not shown). The pLEXA-p100C was used to screen a Jurkat T cell library constructed using pH22AD vector (Human Leukemia MATCHERLEXA cDNA library; Clontech) following the manufacturer’s instructions. Putative positive clones were retransformed to the yeast strain with either the pLEXA-p100C or a nonspecific bait, pLEXA-lamin. After this step of false-positive elimination, specific clones were subjected to DNA sequencing and subsequent BLAST search analyses (www.ncbi.nlm.nih.gov/BLAST/).

Expression Vectors and Antibodies—Expression vectors encoding NIK and NIK mutant, p100, and derivatives, and HA-ubiquitin have been described previously (8, 14a). 100 pmol of siRNA was mixed with 800 ng of pcDNA and transfected into 293 cells were transfected with HA-tagged S9 (0.8 pmol) together with either an empty vector (V), p100 (0.7 µg), or p100 plus NIK (0.5 µg). Cell lysates (250 µg) were subjected to IP using anti-p100, and the associated S9 was identified by IB using anti-HA (top). The cell lysates (7 µg) were also directly subjected to IB using anti-HA (middle) and anti-p100 (bottom) to detect expression levels of S9 and p100, respectively. C, Binding to endogenous p100. 293 cells were transfected with HA-tagged S9 in the presence or absence of NIK, and the cell lysates (500 µg) were subjected to IP with anti-p100 or a nonspecific preimmune serum as a control. Coprecipitated S9 was detected by IB with anti-HA. Cell lysates (14 µg) were also subjected to IB to detect S9 (middle) and p100 (lower) levels. The presence of two background bands (indicated by asterisks) in this experiment was due to the use of more cell lysates and longer exposure of the film in the IB assay to detect the endogenous p100.

RESULTS

Identification of S9 as a p100-Binding Protein—We have previously shown that the C-terminal region of p100 plays a critical role in regulating the processing of p100 (8). To understand the biochemical mechanisms underlying the processing of p100, we performed yeast two-hybrid screens with the C-terminal regulatory region of p100 (amino acids 754–900) fused to the LexA DNA binding domain as bait. We identified five clones encoding different lengths of the same cDNA. BLAST search revealed that this cDNA is identical to a previously cloned gene termed S9 (Fig. 1A). S9 was isolated as a non-ATPase subunit of the 19 S proteasome complex, although its role in protein degradation remained unknown (13). These clones were isolated by yeast two-hybrid screening encode the C-terminal portion of S9 covering the conserved PINT (motif in proteasome subunits, Int-6, Nip-1, and TRIP-15) domain.

To confirm the interaction of S9 with p100 in mammalian cells, we amplified the full-length human S9 cDNA by RT-PCR from Jurkat leukemia T cell cDNA and cloned it into the pcDNA-HA vector (15) downstream of the HA tag. When coexpressed with p100 in 293 cells, S9 weakly interacted with p100, as demonstrated by coimmunoprecipitation of these two proteins by the p100-specific antibody (anti-p100) (Fig. 1B, top lane 2). Under these conditions, S9 was not precipitated by anti-p100 in the absence of transfected p100 (lane 1), suggesting specificity of the coimmunoprecipitation. We then examined the effect of NIK on the S9/p100 interaction, because this kinase serves as a potent inducer of p100 processing (8). As expected, p100 processing was strongly induced in cells expressing NIK (bottom, lane 3). More importantly, the NIK-induced p100 processing correlated with a marked enhancement of the S9/p100 physical interaction (top, lane 3). The level of S9 protein was comparable in the different cell lysates (middle). These results demonstrate that S9 interacts with p100 in
interaction of the DD (19, 20). To further assess the role of the DD in S9 binding, because a p100 mutant (p100-(1–665), see Fig. 2A) lacking the C-terminal 235 amino acids failed to interact with S9 (Fig. 2B, lane 5). Interestingly, another p100 mutant (p100-(1–859)), which lacks the C-terminal 41 amino acids covering its phosphorylation site (Fig. 2A), remained competent in S9 binding (Fig. 2B, lane 4). In fact, this mutant was capable of strong S9 interaction independent of NIK (lane 4 and data not shown). This finding suggests that the sequence located upstream of the phosphorylation site of p100 may serve as an important domain for S9 binding. A visible domain located in this region of interest is the DD. To test the role of the DD in S9 binding, an internal deletion from amino acids 775 to 853 was made to remove the DD from p100. As shown in Fig. 3C, the p100ΔDD was completely inactive in S9 binding in the absence of NIK (lane 3). Furthermore, NIK was unable to induce the binding of this p100 mutant to S9 (lane 4).

The DD present in different proteins is about 90 amino acids in length and characterized by the presence of six α-helices that are essential for maintaining the tertiary structure and function of the DD (19, 20). To further assess the role of the DD in both yeast and mammalian cells and that this molecular interaction can be strongly enhanced by NIK.

To further confirm this interaction, we attempted to visualize the binding using endogenous proteins. Because of the lack of an available S9 antibody, we transfected HA-tagged S9 into 293 cells to observe binding with endogenous p100. Because the level of endogenous p100 is low, it is anticipated that the amount of S9 associated with endogenous p100 will also be low. Therefore, about two times more cell lysates were used than in the experiment shown in Fig. 1B. Interestingly, the anti-p100 antibody could pull down transfected S9, and this interaction was enhanced by NIK (Fig. 1C, lanes 1 and 2). The S9/p100 interaction was specific; S9 could not be pulled down with a nonspecific preimmune serum (Fig. 1C, lane 3). The levels of S9 (middle) and p100 (lower) were comparable in the different samples. Thus, S9 interacts with both endogenous and transfected p100, and this binding is enhanced by NIK.

The DD of p100 Is Essential for S9 Binding to p100—To map the domain of p100 involved in S9 binding, we examined the S9-binding activity of various p100 mutants lacking the C-terminal sequences. Consistent with the yeast two-hybrid assay, the C-terminal region of p100 seemed to be important for S9 binding, because a p100 mutant (p100-(1–665), see Fig. 2A) lacking the C-terminal 235 amino acids failed to interact with S9 (Fig. 2B, lane 5). Interestingly, another p100 mutant (p100-(1–859)), which lacks the C-terminal 41 amino acids covering its phosphorylation site (Fig. 2A), remained competent in S9 binding (Fig. 2B, lane 4). In fact, this mutant was capable of strong S9 interaction independent of NIK (lane 4 and data not shown). This finding suggests that the sequence located upstream of the phosphorylation site of p100 may serve as an important domain for S9 binding. A visible domain located in this region of interest is the DD. To test the role of the DD in S9 binding, an internal deletion from amino acids 775 to 853 was made to remove the DD from p100. As shown in Fig. 3C, the p100ΔDD was completely inactive in S9 binding in the absence of NIK (lane 3). Furthermore, NIK was unable to induce the binding of this p100 mutant to S9 (lane 4).

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FIG. 2. The death domain of p100 is required for S9 binding. A, schematic picture of p100 wild-type (WT) and mutants. RHD, Rel homology domain; ARD, ankyrin repeat domain; P, phosphorylation site. The p100 truncation mutants were named after the amino acid numbers encoded by them. The ΔDD lacks the death domain, whereas the L831P harbors a leucine-to-proline mutation at residue 831. B, co-IP assays to detect the binding of S9 to p100. 293 cells were transfected with HA-tagged S9 together with either an empty vector, p100, p100 plus NIK, or the indicated p100 truncation mutants. The p100 proteins were isolated by IP using anti-p100 followed by detecting the coprecipitated HA-S9 by IB using anti-HA. The death domain of p100 is required for S9 binding. A visible domain located in the C-terminal region of p100 seemed to be important for S9 binding, because a p100 mutant (p100-(1–665), see Fig. 2A) lacking the C-terminal 235 amino acids failed to interact with S9 (Fig. 2B, lane 5). Interestingly, another p100 mutant (p100-(1–859)), which lacks the C-terminal 41 amino acids covering its phosphorylation site (Fig. 2A), remained competent in S9 binding (Fig. 2B, lane 4). In fact, this mutant was capable of strong S9 interaction independent of NIK (lane 4 and data not shown). This finding suggests that the sequence located upstream of the phosphorylation site of p100 may serve as an important domain for S9 binding. A visible domain located in this region of interest is the DD. To test the role of the DD in S9 binding, an internal deletion from amino acids 775 to 853 was made to remove the DD from p100. As shown in Fig. 3C, the p100ΔDD was completely inactive in S9 binding in the absence of NIK (lane 3). Furthermore, NIK was unable to induce the binding of this p100 mutant to S9 (lane 4).

The DD present in different proteins is about 90 amino acids in length and characterized by the presence of six α-helices that are essential for maintaining the tertiary structure and function of the DD (19, 20). To further assess the role of the DD in S9 binding, a proline residue (known to interrupt helix structure) was introduced into the last α-helix of the DD by amino acid substitution. This single amino acid change of leucine 831 to proline (p100L831P) severely affected both the basal and NIK-inducible S9 interaction. This suggested the possibility of an available S9 antibody, we transfected HA-tagged S9 into 293 cells to observe binding with endogenous p100. Because the level of endogenous p100 is low, it is anticipated that the amount of S9 associated with endogenous p100 will also be low. Therefore, about two times more cell lysates were used than in the experiment shown in Fig. 1B. Interestingly, the anti-p100 antibody could pull down transfected S9, and this interaction was enhanced by NIK (Fig. 1C, lanes 1 and 2). The S9/p100 interaction was specific; S9 could not be pulled down with a nonspecific preimmune serum (Fig. 1C, lane 3). The levels of S9 (middle) and p100 (lower) were comparable in the different samples. Thus, S9 interacts with both endogenous and transfected p100, and this binding is enhanced by NIK.

The DD of p100 Is Essential for NIK-induced Post-translational Processing of p100—293 cells were transfected with NIK (0.25 μg) together with p100 (0.8 μg) or p100ΔDD (0.8 μg). At 30 h after infection, the cells were pulse-labeled for 30 min with [35S]methionine/[35S]cysteine followed by IP assays to isolate the radio-labeled p100 proteins. Post-translational generation of p52 was detected from the pulse-labeled wild-type p100 (lane 2) but not from the pulse-labeled p100 lacking the death domain (lane 4).

NIK was also unable to induce the processing of the p100ΔDD, whereas the NIK-inducible p52 was observed in the p100ΔDD (lane 5). Parallel immunoblotting assays showed that the levels of S9 (middle) and the different p100 constructs (bottom) were similar. These results suggest that the DD of p100 serves as an essential domain for its physical interaction with S9.

The DD of p100 Is Essential for NIK-induced Post-translational Processing of p100—we have previously shown that the DD of p100 serves as an inhibitory domain for the constitutive processing of p100 (8); a p100 mutant lacking the DD (p100ΔDD) can undergo constitutive processing. However, the level of this processing is lower compared with that of the NIK-induced p100 processing (Fig. 2C, bottom, lanes 2 and 3; see also Ref. 8). Note that the processing of the p100ΔDD, which is incapable of binding to S9, could not be further enhanced by NIK (lane 4). Interestingly, NIK was also unable to induce the processing of the p100L831P mutant (Fig. 2C, bottom, lane 6), which is also defective for the NIK-inducible S9 interaction. This suggested the possibility

FIG. 3. The death domain of p100 is essential for NIK-induced post-translational processing of p100. 293 cells were transfected with NIK (0.25 μg) together with p100 (0.8 μg) or p100ΔDD (0.8 μg). At 30 h after infection, the cells were pulse-labeled for 30 min with [35S]methionine/[35S]cysteine followed by IP assays to isolate the radio-labeled p100 proteins. Post-translational generation of p52 was detected from the pulse-labeled wild-type p100 (lane 2) but not from the pulse-labeled p100 lacking the death domain (lane 4).
that constitutive and NIK-inducible processing of p100 occur through different biochemical mechanisms and that S9 may be essential for only the latter.

To further explore this possibility, we used an $^{35}$S pulse-chase labeling assay, which allows for the specific detection of post-translational processing. Surprisingly, although the p100ΔDD was shown to constitutively generate p52 by immunoblotting assays, the labeling experiment did not reveal a notable precursor/product relationship after a chase of 2 h, even in the presence NIK (Fig. 3, lane 4). This result indicates that the processing of p100ΔDD either proceeds with slow kinetics or occurs cotranslationally, as has been demonstrated for the constitutive processing of p105 (21). Notwithstanding, mutations in the DD abolish the NIK-inducible post-translational processing of p100, implicating the importance of this domain and interaction with S9 for this biochemical event.

**NIK-induced S9/p100 Interaction Requires p100 Ubiquitination**—Because NIK triggers p100 processing by first inducing its ubiquitination (8), and because ubiquitin conjugation is a common precursor to proteasome association (12), an intriguing possibility is that the ubiquitination of p100 is required for its subsequent binding to S9. To test this idea, we first examined whether the S9/p100 interaction could be enhanced by a catalytically inactive NIK mutant that is incapable of inducing p100 ubiquitination or processing (10). As expected, wild-type NIK markedly promoted the S9/p100 interaction (Fig. 4A, top, lanes 2 and 3), which correlated with the processing of p100 (bottom, lanes 2 and 3). In contrast, the NIK mutant was unable to induce the binding of S9 to p100 (top, lanes 4 and 5), and this was associated with an inability to induce p100 processing (bottom, lanes 4 and 5). Thus, the kinase activity of NIK, and presumably the ubiquitination of p100, is required for the induction of S9/p100 binding.

We further confirmed the requirement for p100 ubiquitination using a genetic approach. We have recently shown that NIK-induced p100 polyubiquitination is mediated by the β-TrCP-containing ubiquitin ligase (10). Suppression of β-TrCP expression by small inhibitory RNA interference blocks NIK-induced p100 ubiquitination and processing. We used this approach to assess whether the ubiquitination of p100 is required for its physical interaction with S9. For these studies, 293 cells were first transfected with either vector alone or the β-TrCP siRNA (lanes 4–6) or a control green fluorescent protein siRNA (lanes 1–3) and then transfected with the indicated cDNA expression vectors. The cell lysates were subjected to IP using anti-p100 followed by IB using anti-HA to detected S9/p100 binding (top). The level of S9 and p100 were analyzed by IB (middle and bottom). C, ubiquitination assay to monitor the requirement of β-TrCP for NIK-induced p100 ubiquitination. The cells were transfected as described in B except for the inclusion of an HA-tagged ubiquitin. After isolation of p100 by IP, the ubiquitin-conjugated p100 adducts (large molecular weight bands) were detected by IB using anti-HA.

**S9 Is Essential for NIK-induced p100 Processing**—Although S9 has been identified as a protein associated with the 19 S proteasome complex, the biological function of this protein remains to be defined. The biochemical studies described above argue for a functional connection between S9 and p100 processing. We investigated this possibility using RNAi-mediated gene suppression, a technique that allows for the transient...
inhibition of specific genes (22). Because the S9 antibody is not available, RT-PCR was used to evaluate the efficiency of the RNAi. As shown in Fig. 5A, transfection of an S9-specific siRNA into 293 cells resulted in marked reduction in the level of S9 mRNA. This inhibition was specific; GAPDH RNA levels were unaffected (Fig. 5A). We also determined the effect of the S9 RNAi on the expression of transfected HA-tagged S9 protein. Consistent with the RNAi analysis, the level of HA-S9 protein was greatly reduced in the RNAi-treated cells (Fig. 5B, lane 2). To further confirm the specificity of this gene suppression approach, we generated a S9 mutant harboring sense mutations at its siRNA binding site. Such mutations do not alter the amino acid codons of S9 but render the S9 mRNA resistant to RNAi-mediated suppression. As expected, the expression of this S9 mutant, termed S9IR, was not affected by the S9-specific RNAi (Fig. 5B, lane 4). We then examined the effect of S9 gene suppression on NIK-induced p100 processing. As shown in Fig. 5C, NIK strongly induced the processing of p100 in the untreated cells, resulting in generation of p52 (lane 2). Interestingly, the inducible p100 processing was markedly inhibited when the expression of S9 was suppressed by the RNAi (Fig. 5C, lane 4). To further confirm that the RNAi-mediated inhibition of p100 processing was caused specifically by the lack of S9, we transfected the RNAi-treated cells with the RNAi-resistant form of S9 cDNA (S9IR). As expected, expression of S9IR efficiently rescued the defect in p100 processing (Fig. 5C, lane 6). Thus, S9 is required for the NIK-induced processing of p100.

**DISCUSSION**

Processing of p100 to generate p52 is a tightly regulated event that is stimulated by phosphorylation at the C-terminal region of p100. We have recently demonstrated that the phosphorylated p100 can be recognized by β-TrCP and targeted for rapid ubiquitination (10). Although the β-TrCP-mediated ubiquitination is essential for p100 processing, the subsequent molecular events of p100 processing remain to be investigated. In the present study, we have shown that p100 can be recognized by the S9 protein located in the “lid” of the 19 S proteasome particle. In mammalian cells, the level of this interaction is strongly enhanced after NIK-induced ubiquitination of p100 (Fig. 4). The mechanism by which ubiquitination promotes p100 binding to S9 remains unclear, but one possibility is that the ubiquitination induces conformational changes in p100, resulting in the exposure of an S9-binding domain, the DD. In support of this possibility, removal of the sequences located downstream of the DD of p100 renders this protein capable of strong interaction with S9 even in the absence of NIK (Fig. 2B). Additionally, deletion of the DD or interruption of one of the α-helices in the DD of p100 largely abrogates the ability of p100 to bind S9 (Fig. 2C).
constitutive p52 generation. Chromosomal translocations of the nfκB2 gene resulting in loss of the C terminus and constitutive p52 levels have been observed in a number of cases of B- and T-cell lymphomas/leukemias (23, 24). The Tax oncoprotein of the human T-cell leukemia virus, an etiologic agent of adult T-cell leukemia, is also a potent inducer of p100 processing, and high levels of p52 have been observed in immortalized T cell lines derived from ATL patients (14). Thus, tight control of p52 levels with the use of multiple checkpoints may be important for proper regulation of cell growth.

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