MOLECULAR AND CELLULAR BIOLOGY, Jan. 2004, p. 475–486 Vol. 24, No. 1
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.1.475–486.2004

Dual Effects of IκB Kinase β-Mediated Phosphorylation on p105 Fate: $SCF^{\beta\text{-}TrCP}$-Dependent Degradation and $SCF^{\beta\text{-}TrCP}$-Independent Processing

Shai Cohen,[1,2] Hillit Achbert-Weiner,[1] and Aaron Ciechanover[1]*

Department of Biochemistry and the Rappaport Family Institute for Research in the Medical Sciences, Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096,[1] and Department of Internal Medicine A, Lady Davis/"Carmel" Medical Center, Haifa 34362,[2] Israel

Received 19 May 2003/Returned for modification 11 July 2003/Accepted 1 October 2003

Processing of the p105 NF-κB precursor to yield the p50 active subunit is a unique and rare case in which the ubiquitin system is involved in limited processing rather than in complete destruction of its target. The mechanisms involved in this process are largely unknown, although a glycine repeat in the middle of p105 has been identified as a processing stop signal. IκB kinase (IKK)β-mediated phosphorylation at the C-terminal domain with subsequent recruitment of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase leads to accelerated processing and degradation of the precursor, yet the roles that the kinase and ligase play in each of these two processes have not been elucidated. Here we demonstrate that IKKβ has two distinct functions: (i) stimulation of degradation and (ii) stimulation of processing. IKKβ-induced degradation is dependent on $SCF^{\beta\text{-}TrCP}$, which acts through multiple lysine residues in the IκBγ domain. In contrast, IKKβ-induced processing of p105 is β-transduction repeat-containing protein (β-TrCP) independent, as it is not affected by expression of a dominant-negative β-TrCP or following its silencing by small inhibitory RNA. Furthermore, removal of all 30 lysine residues from IκBγ results in complete inhibition of IKK-dependent degradation but has no effect on IKK-dependent processing. Yet processing still requires the activity of the ubiquitin system, as it is inhibited by dominant-negative UbcH5a. We suggest that IKKβ mediates its two distinct effects by affecting, directly and indirectly, two different E3s.

The NF-κB family of transcriptional regulators plays important roles in regulating a broad array of basic pathophysiological processes. Among them are the immune and inflammatory responses, development and differentiation, malignant transformation, and apoptosis (9). The p50 and p52 subunits of NF-κB are generated from the inactive precursors p105 and p100, respectively, following limited, ubiquitin- and proteasome-mediated processing. In both cases the C-terminal domain of the molecule is destroyed while the remaining N-terminal part becomes the active subunit (7, 31, 32). Typically, the processed subunits homodimerize or heterodimerize with other members of the rel family of regulators, such as p65 (RelA), RelB, or c-Rel to generate the active homo- or heterodimeric transcription factor. Binding of a member of the IκB family of inhibitors to the dimer generates an inactive trimeric complex that is sequestered in the cytosol. Following stimulation, specific IκB kinases (IKKs) are activated and phosphorylate IκB on specific Ser residues. Phosphorylation leads to recruitment of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase complex, rapid polyubiquitination, and subsequent degradation of the inhibitor by the 26S proteasome. Consequently, active NF-κB can be translocated into the nucleus and initiates specific transcription (19).

The ubiquitin pathway is involved, via specific degradation of short-lived regulatory proteins, in regulation of a broad array of cellular processes. Among these processes are cell cycle progression, differentiation and development, apoptosis, and the immune and inflammatory responses. Degradation of a protein via the ubiquitin system involves two successive steps: (i) formation of a polyubiquitin chain that is covalently anchored to the target substrate, and (ii) degradation of the tagged protein by the 26S proteasome. Conjugation involves activation of ubiquitin by the ubiquitin-activating enzyme, E1, followed by its transfer to a member of the ubiquitin-carrier protein (E2) family of enzymes. In most cases, E2 transfers the activated ubiquitin moiety to a $\varepsilon\text{-}NH_2$ group of an internal lysine residue in the substrate that is specifically bound to an E3, a member of the ubiquitin-protein ligases family of proteins. Subsequent conjugation of additional activated ubiquitin molecules to previously attached moieties generates the polyubiquitin chain that serves as a degradation signal for the 26S proteasome.

The mechanisms involved in limited processing of p105 have been elucidated only partially. Lin and Ghosh have demonstrated that a glycine repeat (also termed the glycine-rich region [GRR]) that spans residues 376 to 404 in human p105 is essential for processing (23) and probably serves as a processing stop signal for the 26S proteasome (30). Several single residues that reside upstream of the GRR and are involved in proper folding of p50 are also essential for processing, most probably via inhibiting unfolding and entry into the proteasome (21). These findings suggest that processing requires at least two motifs, a physical stop signal(s) and a ubiquitina-

* Corresponding author. Mailing address: Department of Biochemistry, Faculty of Medicine, Technion-Israel Institute of Technology, P.O. Box 9649, Haifa 31096, Israel. Phone: 972-4-829-5356. Fax: 972-4-851-3922. E-mail: c_tzachy@netvision.net.il.

tion-E3 recognition site. As for regulation of generation of p50, processing appears to proceed via two independent mechanisms: (i) basal-constitutive and (ii) signal induced. Fan and Maniatis (7) have shown that a truncated form of p105, p60, can still be processed to p50. Lin and colleagues (22) have shown that p105 can be processed cotranslationally, and synthesis of the complete molecule is not required for generation of p50. Because the signal-induced E3 recognition motif resides within the C-terminal domain of the molecule, these studies imply that all the motifs that are required for processing in the resting cell are contained within the first ~550 amino acid residues. Other studies have suggested a role for phosphorylation of the C-terminal domain of p105 in regulated, signal-induced processing of the molecule (10, 24), though here it appears that some of the p105 molecules are completely destroyed. Heissmeyer and colleagues (15) have shown that IKK-mediated phosphorylation of Ser residues that reside in a sequence that spans amino acid residues 922 to 933 leads to rapid degradation of p105. It was recently shown that this IKK-mediated phosphorylation leads to recruitment of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase. Consequently the molecule is ubiquitinated and rapidly processed, with a certain proportion being completely degraded (29). Heissmeyer and colleagues later reported similar findings (14). While the structural motives and ubiquitin system enzymes involved in basic-constitutive processing are not known, this process appears to require an additional adjacent downstream domain that contains lysine residues 440 and 441 (which are probably important for ubiquitination) and an acidic region (residues 445 to 453) that may function as an E3 recognition motif (30).

Interestingly, processing of p100, the gene product of *nfκb2*, to yield the p52 subunit is mediated by a similar mechanism. Like p105, part of it may occur cotranslationally and requires the GRR (18). A recent study has demonstrated that phosphorylation of Ser residues 867 and 870 that is mediated by IKKα is required for processing (35). It is interesting that the phosphorylation sites of IKK in p105 and p100 are similar to those of IκBα, β-catenin, and human immunodeficiency virus Vpu, where the two critical serines are interspaced by three residues.

The C-terminal segment of p105 that resides between the GRR and the IKK/TrCP motif contains seven ankyrin repeats. Active NF-κB subunits, such as p50 and p65, dock to this region and inhibit further processing of the precursor. This results in sequestration of the docked subunits as an inactive form in the cytosol. Thus, the ankyrin repeat domain serves as an inhibitor of NF-κB activity (13, 33, 36). In agreement with these findings, Harhaj and colleagues have shown that constitutive processing of newly synthesized p105 molecules, to which p50 and p65 are not yet docked, is more efficient than that of older molecules that are already associated with p50 and p65 (12). Accelerated, signal-induced processing or degradation of p105 leads to release of the docked active factors (5, 15, 29) with their subsequent translocation to the nucleus.

However, as noted, IKKβ and β-TrCP expression also lead to degradation of a certain proportion of the p105 precursor molecules (5, 15), and it was important to identify the role of the kinase and ligase in regulating the two distinct processes. Here we demonstrate that IKKβ mediates both processing and degradation of p105, and the two functions require the C-terminal phosphorylation domain. In contrast, β-TrCP is involved only in degradation of the molecule. However, processing still requires an intact ubiquitin system and probably ubiquitin ligase E3.

MATERIALS AND METHODS

Materials. Materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Bradford reagent were from Bio-Rad. L-[^{35}S]methionine (>1,000 Ci/mmol; ~50 mCi/ml) for in vitro translation, an L-[^{35}S]methionine and L-[^{35}S]cysteine mixture (>1,000 Ci/mmol; ~15 mCi/ml) for metabolic labeling, and prestained molecular weight markers were obtained from Amersham Pharmacia Biotech. For site-directed mutagenesis we used the QuickChange kit from Stratagene. Tissue culture sera and media were from Biological Industries, Bet Haemek, Israel, or from Sigma. Rabbit anti-NF-κB1 p50 antibody that recognizes both p105 and p50 was from Santa Cruz, and peroxidase-conjugated goat anti-rabbit antibody was from Jackson ImmunoResearch Laboratories. Ubiquitin, dithiothreitol, ATP, adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS), phosphocreatine, creatine phosphokinase, 2-deoxyglucose, glutathione, glutathione immobilized to agarose beads, isopropyl β-D-thiogalactopyranoside, deoxycholic acid, and Tris buffer were from Sigma. Lactacystin β-lactone, HEPES buffer, and protease inhibitors mixture were from Calbiochem. Hexokinase and Fugene 6 transfection reagent were from Roche Molecular Biochemicals. Oligofectamine reagent was from Invitrogen. Methylated ubiquitin (MeUb) and ubiquitin aldehyde (UbAl) were from Affiniti Research Products. Reagents for enhanced chemiluminescence (ECL) were from Pierce. The wheat-germ-extract-based coupled transcription-translation kit was from Promega. Restriction and modifying enzymes were from New England Biolabs. Oligonucleotides were synthesized by Biotechnology General, Rehovot, Israel, or by Sigma. siRNA was synthesized by Dharmacon Research, Inc. All other reagents were of high analytical grade.

Cell lines. COS-7, HEK 293, and HeLa cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (penicillin-streptomycin).

Plasmids and construction of mutants. The human p105-WT, p105-Δ917-933 (Fig. 1a and b, respectively), and p105-TthIII 1 (p105 to 544) (the TthIII site is shown on the p105-WT scheme in Fig. 1a and e) cDNA constructs used for in vitro translation (in pT7β105) and for transient transfection (in pCI-neo; Promega) in cells were described previously (29–31) and served as a base for further manipulations and expression. The p105 N-terminal truncated mutants (577-968/END and 660-968/END; Fig. 1c and d, respectively) were generated by PCR. We mutated or deleted all 30 lysine residues downstream of the GRR (downstream of lysine residue 425) in the IκBγ C-terminal domain of the p105 molecule (the cleaved domain). All K-to-R substitutions were generated by site-directed mutagenesis by using the QuikChange kit. Each replaced or deleted lysine was designated by a serially increasing number as described in Table 1. Initially, lysine residues 18 to 30 were sequentially replaced by arginine (the last construct, p105-K18-30R, in which all 13 lysine residues were replaced, is shown in Fig. 1e). Lysine residues 2 to 17 were removed via deletion of entire regions in the p105 molecule. Thus, p105-Δ544-654/ΔK12-17 and p105-Δ429-654/ΔK2-17 (depicted in Fig. 1f and g, respectively) were generated by PCR insertion of a second *Bsm*I site (in positions 544 and 429, respectively; the endogenous site is in position 654), digestion with *Bsm*I, and religation. In addition, we constructed a mutant that lacks lysine residues 12 to 30 (p105-Δ544-654/ΔK12-17;K18-30R; Fig. 1h) and lysine residues 1 to 30 (p105-Δ429-654/ΔK2-17;K1,18-30R; here we also replaced K1 with R; Fig. 1i). Initially, all mutations were carried out by using p105-WT in the pT7β105 vector. For expression of proteins in COS-7 or 293 cells the entire coding frames from the desired p105 mutants were subcloned from the pT7β105 vector into the *Xho*I and *Not*I sites in the pCI-neo vector by using PCR and *Xho*I and *Not*I sites that were introduced into the cDNA. Constitutively active IKKβ (IKKβ-SS>EE) and ΔF-box human β-TrCP1 (ΔF-β-TrCP1) for cell expression were as described previously (29). Active-site mutants, dominant-negative species of the E2 enzymes UbcH5a (C85A) and UbcH5c (C85A) for cell expression, were as described previously (11). Sequence of all constructs was confirmed by using the Applied Biosystems ABI PRISM 3100 autosequencer.

Generation of radiolabeled and recombinant proteins. [^{35}S]methionine-labeled p105 proteins for experiments in a cell-free system were synthesized by using the wheat-germ-based coupled transcription-translation kit according to the manufacturer's instructions. A 6-His-tagged β-TrCP1-containing SCF complex (h-β-TrCP1 · Skp1 · cullin1 · Rbx1/Roc1) was generated by using a baculovirus expression system as described previously (29). Baculovirus-expressed constitutively active IKKβ was as described previously (27).

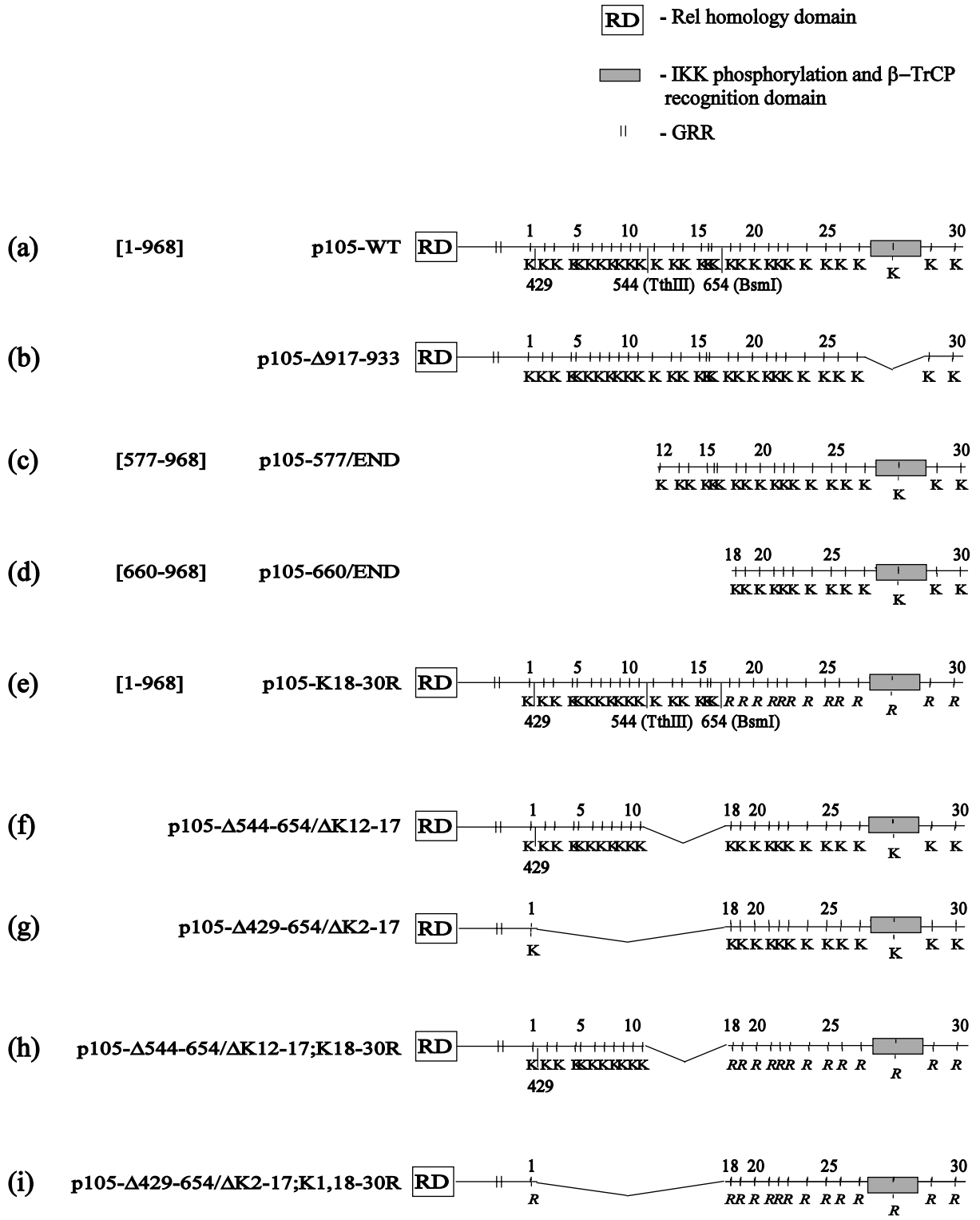

FIG. 1. Schematic representation of p105-WT and the different p105 mutants. The different p105 proteins (a to i) were constructed and designated as described in Materials and Methods and in Table 1. Numbers in square brackets denote the amino acid residue number in the protein sequence. Numbers above the scheme line mark, in serially increasing numbers, all lysine residues that reside downstream of the GRR (downstream of lysine 425 [denoted 1]). The site of important restriction enzymes is marked (amino acid residue and enzyme name) in panels a and e. The different domains of p105 (RD, IKK phosphorylation and β-TrCP recognition sites, and the GRR) are marked and annotated in the figure body.

TABLE 1. Lysine residue numbers in the IκBγ domain of p105[a]

Designated serial no. of lysine residue	Residue no. in p105
1	425
2	431
3	440
4	441
5	448
6	452
7	460
8	490
9	504
10	510
11	526
12	593
13	624
14	629
15	636
16	638
17	639
18	683
19	739
20	791
21	809
22	816
23	821
24	830
25	838
26	856
27	896
28	935
29	953
30	967

[a] The residue number along the protein sequence of all 30 lysine residues in the IκBγ domain of p105 are shown with an increasing serial number (1 to 30) assigned to each of these residues to facilitate identification.

Preparation of whole-cell, nuclear, and cytoplasmic extracts. HeLa cell extract was prepared by hypotonic lysis as described previously (29, 30). Nuclear and cytoplasmic extracts were prepared by hypotonic lysis and Nonidet P-40 extraction (cytosol), followed by treatment with a high glycerol-high salt nuclear extraction buffer as described previously (1).

In vitro conjugation assays. Adducts of ubiquitin with p105-WT or the various p105 K-to-R mutants were synthesized as described previously (29). Briefly, in vitro-translated and [^{35}S]methionine-labeled proteins were phosphorylated in a cell-free system in a reaction mixture containing (in a total volume of 4.5 μl) 2.5 μl (~20,000 cpm) of the labeled substrate (see above), 5 mM $MgCl_2$, 0.5 mM ATP, 1 μM okadaic acid, and baculovirus-expressed IKKβ (~0.4 μg where indicated). Reaction mixtures were preincubated for 20 min at 30°C. Where indicated, $SCF^{\beta\text{-}TrCP1}$ complex was added (~1.5 μg) along with bacterially expressed purified recombinant UbcH5c (0.5 μl) (11) and purified, baculovirus-expressed E1 (250 ng) (20). In other cases the phosphorylated (or nonphosphorylated) proteins were incubated in a complete HeLa cell extract (100 μg). All these reactions also contained (in a total volume of 12.5 μl and as indicated) 0.5 μg of UbAl, a specific inhibitor of certain isopeptidases (17), MeUb (12.5 μg), and ATPγS (5 mM). Reaction mixtures without ATP contained 20 mM 2-deoxyglucose and 0.2 μg of hexokinase instead of ATPγS. Following incubation (45 min at 37°C), reaction mixtures were resolved by SDS–10% PAGE. Gels were dried and proteins were visualized by using a PhosphorImager (Fuji).

siRNA. Double-stranded siRNA to silence human β-TrCP1 and -2 was as described previously (8). The sequences are GUG GAA UUU GUG GAA CAU CTT (sense) and GAU GUU CCA CAA AUU CCA CTT (antisense). Transfection of 293 cells with the siRNA oligonucleotides was performed by using Oligofectamine reagent following the manufacturer's instructions. Briefly, 293 cells were seeded into 6-well plates (35-mm diameter) 16 to 18 h prior to transfection. β-TrCP siRNA (~0.2 nmol) was transfected into cells. Forty-eight hours following transfection, DNA expression vectors encoding p105-WT and constitutively active IKKβ were transfected into the cells. Cell extracts and RNA were prepared 24 h after DNA transfection. The efficacy of β-TrCP gene suppression was monitored by real-time PCR. Specific RNA levels decreased by ~85%.

Transient transfections and processing or degradation of p105 in intact cells. COS-7 cells were transiently transfected with ~1.5 μg of a cDNA coding for the wild type (WT) or one of the various p105 mutant or deleted proteins. Where indicated, cells were also cotransfected with ~1.5 μg of each cDNA coding for the constitutively active IKKβ, ΔF-β-TrCP1, and the dominant-negative UbcH5c or UbcH5a (DN-UbcH5c or DN-UbH5a, respectively). An empty vector was added, when necessary, to maintain an equal amount of DNA in all transfections. Transfection was carried out by using the Fugene reagent following the manufacture's instructions. Twenty-four hours after transfection, processing and degradation of p105 in cells was monitored by either pulse-chase labeling followed by immunoprecipitation and detection of the proteins by phosphorimaging or via Western blot analysis as described below. For pulse-chase labeling and immunoprecipitation, cells (growing in 60-mm-diameter dishes) were washed twice in Hank's balanced salt solution and were incubated (60 min per pulse) in 1 ml of medium lacking methionine and cystine but containing the [^{35}S]methionine-cystine mixture (50 μCi). Following labeling, cells were harvested (pulse, time zero) or were washed in a complete medium containing a 2 mM excess of unlabeled methionine and cystine, and incubation continued for an additional 2 h in the same medium (chase). Following lysis in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 0.5% deoxycholic acid, 50 mM Tris [pH 7.6], 0.1% SDS, and protease inhibitors mixture), labeled proteins were precipitated with anti-p105 antibody. Immune complexes were collected with immobilized protein A and were resolved by SDS–10% PAGE. Proteins were then visualized with a PhosphorImager. For immunoblotting, cells were lysed either in RIPA buffer (described above) or, where indicated, in cytosolic and nuclear fractions prepared as described above. Aliquots representing an equal number of cells were resolved by SDS–10% PAGE and were blotted onto nitrocellulose paper. Processing and degradation of p105 was monitored via Western blot analysis by using anti-p50 antibody and ECL.

Protein concentration. Protein concentration was determined by the method of Bradford (4) by using bovine serum albumin as a standard.

RESULTS

Identification of the lysine residues involved in signal-induced and $SCF^{\beta\text{-}TrCP}$-mediated ubiquitination of p105. IκBα is ubiquitinated, following signaling, on two lysine residues, 21 and 22 (2, 34), that reside 10 residues upstream of the IKKβ phosphorylation and E3-binding sites of the molecule (Ser32 and Ser36). Since IκBα degradation and p105 processing-degradation utilize similar signal-induced structural motifs (14, 15, 29) that serve the same purpose of providing the cell with active NF-κB, it was important to compare not only the phosphorylation domains in the two molecules but also the ubiquitin-anchoring sites. Since p105, unlike IκBα, does not contain two adjacent lysine residues that reside upstream and in the neighborhood of the phosphorylation site, we assumed that their identification will not be straightforward. Accordingly, we wanted to first test the hypothesis that the lysine residues that are ubiquitinated following signaling reside in the C-terminal domain of the molecule. To that end we constructed two deletion mutants that span the IκBγ domain of p105, p105-577/END(577-968) and p105-660/END(660-968). As can be seen in Fig. 2 and as is the case for p105-WT that is ubiquitinated following IKK-mediated phosphorylation (Fig. 2A), the two deletion mutants are also ubiquitinated efficiently (Fig. 2B and C). Ubiquitination requires both the phosphorylation and E3 anchoring site in p105 (Ser residues 923, 927, and 932) as well as $SCF^{\beta\text{-}TrCP}$ (Fig. 2A and B) and IKKβ (Fig. 2C). Since p105-660/END(660-968) is ubiquitinated efficiently, we sought to first identify the targeted lysine residues in this domain. Accordingly, we replaced sequentially lysine residues 18 (683) through 30 (967) in the WT p105 molecule. As can be seen in Fig. 3A and B, there is no single lysine residue that serves as a

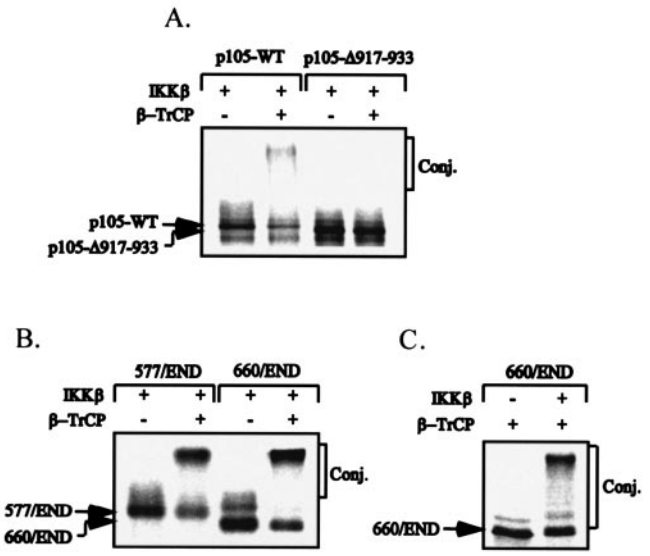

FIG. 2. Truncated p105 proteins that span the IκBγ domain but lack the p50 portion of the molecule are efficiently ubiquitinated following phosphorylation. (A) In vitro-translated and [^{35}S]methionine-labeled IKKβ-phosphorylated WT and p105-Δ917-933 were subjected to in vitro conjugation in a reconstituted cell-free system as described in Materials and Methods. SCF$^{\beta\text{-TrCP}}$ was added where indicated. (B) [^{35}S]methionine-labeled p105 mutants p105-577/END(577-968) and p105-660/END(660-986) were subjected to phosphorylation and ubiquitination as described in Materials and Methods and in the legend to panel A. (C) [^{35}S]methionine-labeled p105-660/END(660-968) was preincubated in the absence or presence of IKKβ, and incubation continued in the presence of SCF$^{\beta\text{-TrCP}}$. Proteins were resolved by SDS-PAGE and were visualized by exposure to a PhosphorImager screen. Conj., conjugates.

major ubiquitination site, and we observed a gradual decrease of conjugation with increasing numbers of lysine residues replaced. This decrease started to be apparent only when we substituted more than six residues. Altogether, conjugation was decreased by 80%. Thus, it can be concluded that most of the lysines targeted by β-TrCP reside among these residues. Further corroborating this notion is the experiment shown in Fig. 3C. Here we utilized MeUb rather then ubiquitin in a cell-free reconstituted conjugation assay. This derivative is unable to generate the polymerized ubiquitin chain and singly modifies the targeted lysine residue (16). As is clearly seen, the resolved WT protein contains several distinct conjugates, most probably representing monoubiquitin derivatives of multiple lysine residues in the same molecule. Their numbers decrease gradually with increasing numbers of lysine residues replaced.

Since p105-K18-30R can still be conjugated, although to a significantly lower degree than that of the WT (20%), it was important to test whether substitution of the 13 lysines affected processing and generation of p50. As can be seen in Fig. 4A, the presence of active IKK leads to almost complete disappearance of both p105-WT and p105-K18-30R and to the generation of a similar amount of p50 from these proteins. Thus, it appears that removal of these lysine residues does not have a major effect on either degradation or processing of the precursor protein. As was noted earlier (5), IKK-mediated degradation of p105 results in disappearance of the cytosolic anchor of free p50 that now migrates to the nucleus (Fig. 4A, compare lane 5 to lane 3 and lane 9 to lane 7). Similar results were obtained by using pulse-chase labeling and immunoprecipitation (Fig. 4B). Here IKKβ expression resulted in almost complete disappearance of the precursor WT and the mutant with lysines 18 to 30 replaced (compare p105 in lanes 3 and 4 to that in lanes 7 and 8), accompanied by stimulation-induced generation of p50. It should be noted that generation of p50 was slightly affected by the extensive lysine replacement (compare lane 8 to lane 4). It is important to note that both degradation and processing are dependent on an intact IKK phosphorylation and β-TrCP anchoring domain at the C-terminal region of the molecule (Fig. 4B, lanes 9 to 12). We note, as did Heissmeyer and colleagues (14), that expression of IKKβ suppresses the basal, signal-independent processing of p105 that lacks the phosphorylation domain. This is possibly due to the fact that IKK binds to the substrate in the death domain which resides upstream of the phosphorylation site. In the mutant p105 the kinase cannot catalyze phosphorylation and may remain bound to the substrate, thus hindering access to p105 by either the conjugation machinery (E3) involved in basal processing or the proteasome.

Of note is that when Western blot detection (in contrast to pulse-chase analysis; see below) is used (Fig. 4A), quantitative analysis of the p50 generated in IKKβ-expressing cells exceeds the amount of p105 that is present in the cells (compare the total amount of p50 in lanes 4 and 5 and in lanes 8 and 9 to the total amount of p105 in lanes 2 and 4 and in lanes 6 and 8). As noted, this discrepancy is not seen when we use pulse-chase analysis (Fig. 4B). The reason is that during the 24 h of transfection in the presence of IKKβ that precedes the analysis, the synthesized p105 is rapidly processed and degraded under the effect of the kinase, and a stable p50 is accumulated continuously.

Since substitution of lysine residues 18 to 30 only slightly affected the stimulation-dependent degradation and processing of p105, we decided to remove additional lysine residues. Accordingly, we deleted a segment of p105 that contains lysine residues 12 (593) to 17 (639) (p105-Δ544-654/ΔK12-17) and a segment that contains residues 2 (431) to 17 (639) (p105-Δ429-654/ΔK2-17). As expected, these two mutants are ubiquitinated efficiently by β-TrCP (Fig. 5A, compare lanes 4 and 6 to lane 2), as lysine residues 18 (683) to 30 (967) have remained intact. Thus, we generated additional p105 species that lack lysine residues 12 (593) to 30 (967) (p105-Δ544-654/ΔK12-17; K18-30R) and 1 (425) to 30 (967) (p105-Δ429-654/ΔK2-17: K1,18-30R). The last protein does not contain a single lysine residue in the IκBγ domain (downstream of the GRR). As can be seen in Fig. 5A, elimination of lysine residues 12 to 30 inhibits conjugation dramatically (~90%) (compare lane 8 to lanes 2, 4, and 6), while elimination of all 30 residues inhibits the modification altogether (Fig. 5A, lane 10). We tested the ability of these p105 mutants to undergo processing. As can be seen in Fig. 5B, the mutant that lacks lysine residues 12 to 30 undergoes efficient IKK-dependent processing (Fig. 5B, lanes 5 to 8), similar to the WT protein (data not shown), and to a mutant p105 that lacks residues 12 to 17 (Fig. 5B, lanes 1 to 4). However, in striking contrast to the WT and p105-Δ544-654/ΔK12-17, the mutant that lacks lysine residues 12 to 30 is significantly more resistant to degradation (Fig. 5B, compare lanes 7 and 8 to lanes 3 and 4). Thus, for the first time we were

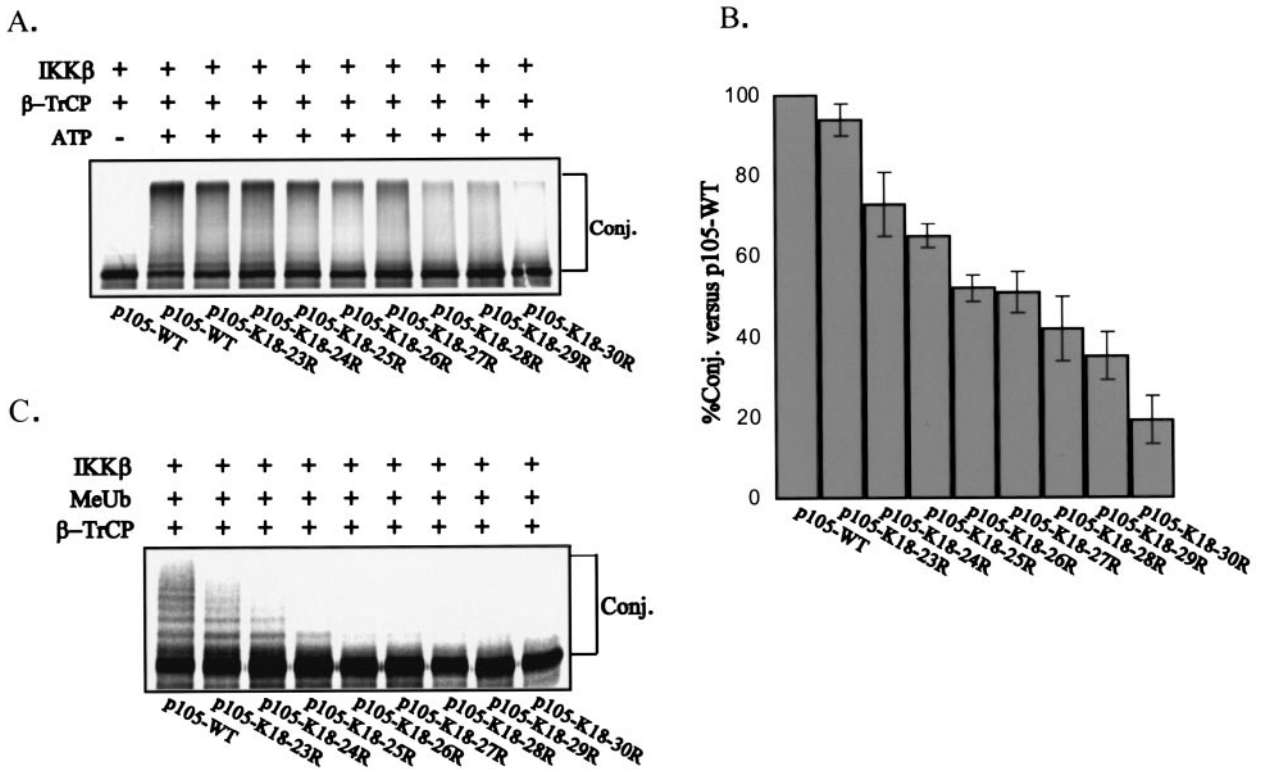

FIG. 3. Multiple p105 C-terminal lysine residues are targeted by β-TrCP. In vitro-translated, L-[^{35}S]methionine-labeled, and IKKβ-phosphorylated WT and C-terminal K-to-R p105 mutants (see Table 1) were subjected to in vitro conjugation in a reconstituted cell-free system as described in Materials and Methods. Proteins were resolved by SDS-PAGE and were visualized by using a PhosphorImager. (A) Labeled p105-WT and the different K18-30R mutants (starting from p105-K18-23R; Table 1) were subjected to conjugation and were resolved by SDS–10% PAGE. (B) Data from three independent experiments, with the error bar representing one standard deviation, are presented. Percent conjugation is the ratio between radioactivity in the conjugates divided by the sum of the radioactivity in the conjugates and that in the remaining unreacted protein. (C) Reaction mixtures were similar to those described for panel A, except that ubiquitin was replaced with MeUb, and proteins were resolved via SDS–15% PAGE. Conj., conjugates.

able to discover two apparently distinct functions of IKK, stimulation of degradation and stimulation of processing. Figure 5C describes a similar experiment in which we used a pulse-chase labeling and immunoprecipitation experimental approach rather than Western blot analysis. This approach allows a quantitative measurement, demonstrating clearly that removal of lysine residues 12 to 30 does not affect processing but inhibits degradation almost 10-fold (Fig. 5C; compare lanes 4 to 8). Figure 5D demonstrates that removal of all 30 lysine residues from IκBγ results in complete inhibition of IKK-dependent degradation but has no effect whatsoever on IKK-dependent processing (compare degradation in Fig. 5D, lane 12, to that in lane 4 and lane 8), demonstrating that the two functions of the kinase are mechanistically distinct.

$SCF^{\beta\text{-}TrCP}$ is required for degradation but not for processing of p105. As our results indicate (see above), systematic mutational replacement of all lysine residues in the IκBγ domain of p105 revealed two distinct functions of IKKβ in p105 dynamics: stimulation of degradation and stimulation of processing. Thus, it was important to test whether the ubiquitin ligase β-TrCP catalyzes both or whether it is involved selectively in only one process. As can be seen in the experiment depicted in Fig. 6A, inhibition of the enzyme activity by either expressing a dominant-negative protein (lane 4; ΔF-box β-TrCP; this protein cannot bind the Skp1 component of the SCF ligase complex and therefore cannot recruit the E2 enzyme, yet the ΔF-box β-TrCP can still bind the substrate and stabilize it) or, following its silencing by siRNA (lane 5), inhibits stimulation-induced degradation of the p105 precursor completely. In contrast, the inhibition of β-TrCP had no effect on the generation of p50. Taken together from the experiments described in Fig. 5B, C, D, and 6A, it appears that β-TrCP-dependent degradation of the precursor is mediated by the lysine residues in the IκBγ domain. Indeed, as can be clearly seen in Fig. 6B, ΔF-box β-TrCP inhibits IKK-dependent degradation of p105-WT (lanes 2 to 4) and p105-Δ544-654/ΔK12-17 (lanes 5 to 7) (compare p105 in lanes 4 and 3 and in lanes 7 and 6). Since IKK does not affect the disappearance of p105-Δ544-654/ΔK12-17;K18-30R (lanes 8 and 9; this protein lacks lysine residues 12 to 30) and p105-Δ429-654/ΔK2-17; K1,18-30R (lanes 10 and 11; this protein lacks all lysine residues in the IκBγ domain, lysines 1 to 30), it is expected that the dominant-negative β-TrCP will have a minimal effect or no effect at all on the degradation of these two species, which was indeed the case (data not shown).

An active ubiquitin system is required for IKK-mediated processing of p105. At this point it was important to test whether processing and degradation are both proteasome de-

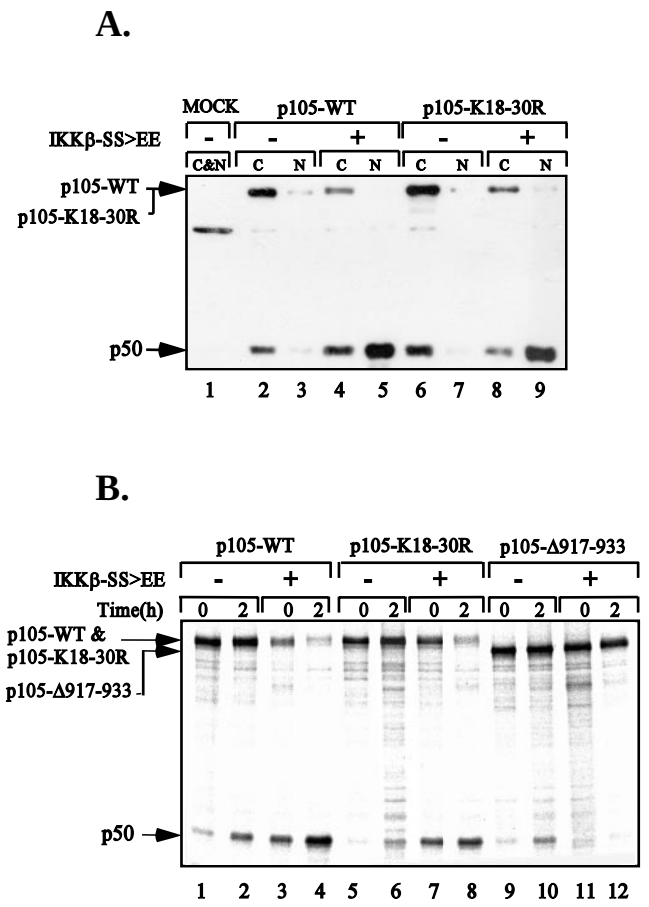

FIG. 4. Stimulation-induced processing and degradation of p105 is not affected by mutating lysine residues 18 (683) to 30 (967) in the C-terminal domain of the molecule. (A) COS-7 cells were transiently transfected with either a cDNA coding for p105-WT (lanes 2 to 5) or p105-K18-30R (lanes 6 to 9) as indicated. Control cells (lane 1) were transfected with an empty vector. Where indicated, a cDNA coding for constitutively active IKKβ was cotransfected. Twenty-four hours after transfection cells were harvested, and nuclear and cytosolic fractions were isolated as described in Materials and Methods. Aliquots of cytosolic and nuclear extracts representing an equal number of cells were resolved via SDS–10% PAGE and were blotted onto nitrocellulose paper, and proteins were visualized by using anti-p50 antibody and ECL as described in Materials and Methods. C, cytosolic fraction; N, nuclear fraction. (B) COS-7 cells were transiently transfected with a cDNA coding for either p105-WT (lanes 1 to 4), p105-K18-30R (lanes 5 to 8), or p105-Δ917-932 (lanes 9 to 12). Where indicated, cDNA coding for a constitutively active IKKβ was cotransfected. Twenty-four hours after transfection cells were pulse labeled with [^{35}S]methionine (0; pulse). Following removal and dilution of the labeled amino acid and further incubation for 2 h (2; chase), the labeled proteins were immunoprecipitated by using anti-p50 antibody, resolved by SDS–10% PAGE, and visualized by phosphorimaging as described in Materials and Methods.

pendent and, more importantly, whether processing is also mediated by the ubiquitin system. Since elimination of all lysine residues in the IκBγ domain did not affect IKK-dependent processing (Fig. 5D and 6B) and since processing did not require the β-TrCP ubiquitin ligase (Fig. 6), it was important to test whether the process is at all dependent on an active ubiquitin system, particularly in light of the finding that stimulated processing does require both IKKβ and its p105 phosphorylation site (which is also the β-TrCP binding site) (Fig. 4B). As can be seen in Fig. 7A, both basal and stimulated processing of p105 as well as stimulation-induced degradation of p105 require an active proteasome: inhibition of the proteasome eliminates the two modes of generation of p50 as well as degradation of p105 (in Fig. 7A, compare p50 in lanes 2 and 4 to p50 [or the lack thereof] in lanes 6 and 8 and compare p105 in all lanes, but mostly in lanes 4 and 8). By using different dominant-negative species of E2s (cysteine-to-alanine mutants in the ubiquitin binding site; these enzymes cannot bind ubiquitin, but they can bind to the E3 enzyme that cannot conjugate ubiquitin to its bound substrate), we were able to show that the ubiquitin system is involved in both stimulation-induced processing and degradation. As can be seen in Fig. 7B (compare p105 in lane 3 to that in lane 2), DN-UbcH5c selectively inhibits degradation of p105, similar to the effect of β-TrCP (Fig. 6). In contrast, DN-UbcH5a blocks both processing and degradation (compare p105 and p50 in lane 4 to those in lanes 2 and 3). These data reflect steady-state levels of p105 and p50 in transfected cells as determined by Western blot analysis. Similar corroborating results were obtained by using a pulse-chase labeling and immunoprecipitation experimental approach (Fig. 7C). It should be stressed that these E2 enzymes affect only stimulation-induced processing and degradation and not basal, nonstimulated processing; as can be seen in Fig. 7D, p105-TthIII (p105 1 to 544), a truncated species of p105 that is longer than p50 (containing 544 residues versus 435 residues of p50), is processed in cells in the presence of either DN-UbcH5c or DN-UbcH5a.

As we have shown, replacement of all lysine residues in the IκBγ domain of p105 completely blocked degradation of p105 but had no effect on processing (see, for example, Fig. 5D). This finding raised an interesting possibility that the lysine residues required for degradation are different from those required for processing. The first step in corroborating such a hypothesis is to demonstrate that p105 protein that harbors a lysine-less (1 to 30) IκBγ C-terminal domain cannot be conjugated by β-TrCP, yet a crude extract can catalyze its ubiquitination, probably via a different E3. The results of the experiment depicted in Fig. 8 show that this is indeed the case. The experiment depicted in panel A demonstrates that while β-TrCP conjugates p105-WT efficiently (lanes 1 and 2), the conjugation of p105 that lacks lysine residues 12 to 30 is severely affected (lanes 3 and 4) while that of a p105 species in which all the lysine residues (1 to 30) have been replaced is completely inhibited (lanes 5 and 6). The experiment depicted in panel B demonstrates that in the absence of IKK there is no conjugation of the lysine-less (1 to 30) species even when the reaction is carried out in HeLa extract that apparently contains all the ubiquitin ligases required for p105 metabolism. The IKK-independent conjugation observed mostly with the WT species is due to basal, signal-independent activity leading to p105 processing (31, 32). Importantly, however, when IKK is added to the HeLa extract (Fig. 8C) the lysine-less (1 to 30) p105 is conjugated (lane 6), unlike its counterpart that was incubated in the presence of purified β-TrCP (Fig. 8A, lane 6). This result strongly suggests that active IKK and the IKK phosphorylation site in p105 are required for the activity of a yet-to-be-identified E3 involved in stimulation-dependent p105 processing.

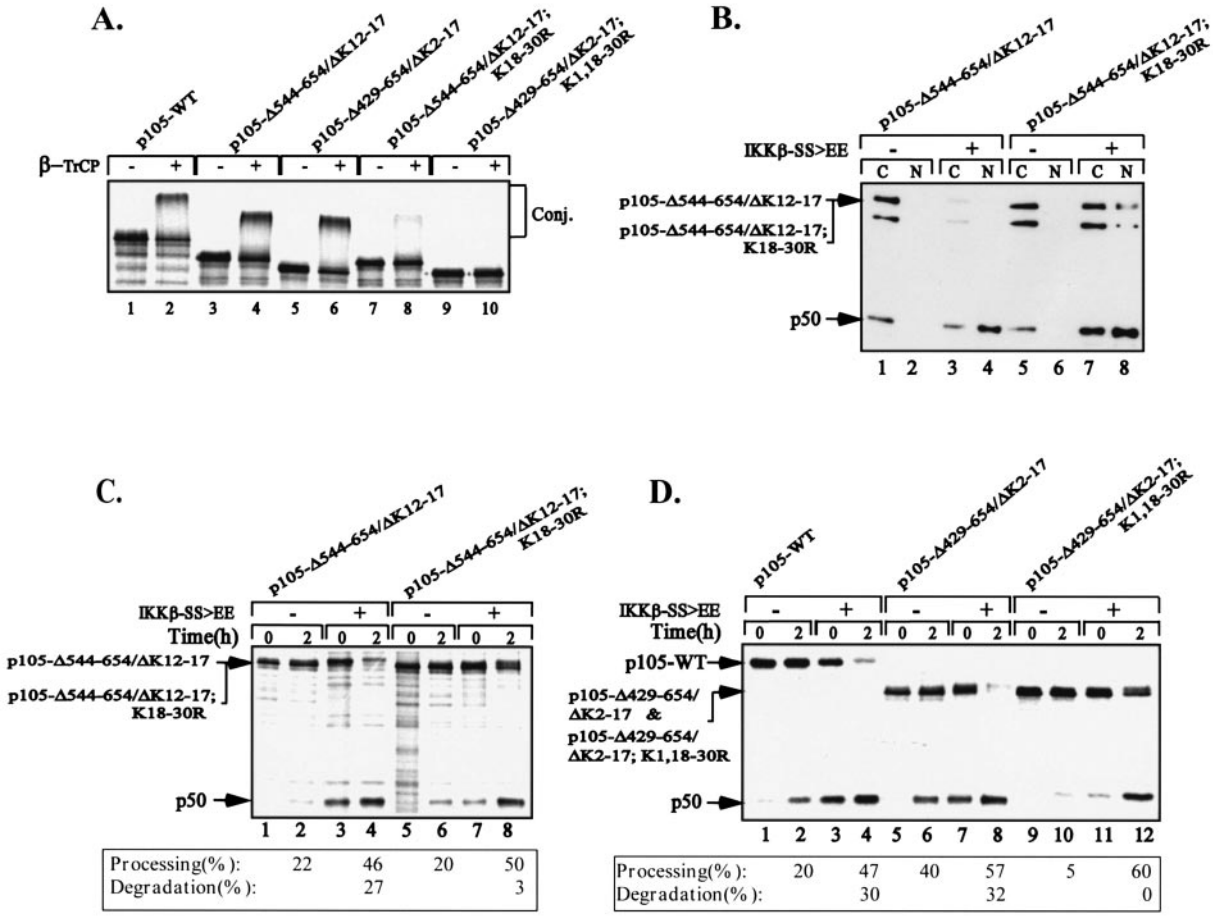

FIG. 5. Removal of p105 lysine residues 12 to 30 reveals two distinct roles for IKK in p105 activation: stimulation of degradation and, independently, stimulation of processing. (A) Removal of lysine residues 1 (425) to 30 (969) completely inhibits conjugation of p105. In vitro-translated, [^{35}S]methionine-labeled, and IKKβ-phosphorylated WT-p105 as well as p105 species that lack lysine residues 12 to 17 (p105-Δ544-654/ΔK12-17), 2 to 17 (p105-Δ429-654/ΔK2-17), 12 to 30 (p105-Δ544-654/ΔK12-17;K18-30R), and 1 to 30 (p105-Δ429-654/ΔK2-17;K1,18-30R) were subjected to SCF$^{\beta\text{-TrCP}}$-mediated conjugation in a cell-free system as described in Materials and Methods and in the legends to Fig. 2 and 3. Proteins were resolved by SDS–10% PAGE and were visualized by exposure to a PhosphorImager screen. Conj., conjugates. (B) Removal of p105 lysine residues 12 to 30 inhibits IKK-stimulated degradation but does not affect IKK-induced processing (Western blot analysis). COS-7 cells were transiently transfected with a cDNA coding for p105-Δ544-654/ΔK12-17 (lacking lysine residues 12 to 17; lanes 1 to 4) or p105-Δ544-654/ΔK12-17;K18-30R (lacking lysine residues 12 to 30; lanes 5 to 8). Where indicated, a cDNA coding for constitutive active IKKβ was cotransfected. Twenty-four hours after transfection cells were harvested and p105 and p50 were detected by Western blot analysis in nuclear and cytoplasmic fractions as described in Materials and Methods and in the legend to Fig. 4A. (C) Removal of p105 lysine residues 12 to 30 reduces IKK-stimulated degradation but does not affect induced processing (analysis by pulse-chase labeling and immunoprecipitation). COS-7 cells were transiently transfected for 24 h with cDNAs coding for the different species of p105 mutants and IKKβ as described in the legend to panel B. Processing and degradation of the p105 mutants was monitored in pulse-chase labeling and immunoprecipitation experiments as described in Materials and Methods and in the legend to Fig. 4B. Quantified data are presented. Percent of processing was calculated with the following equation: [(p50 detected at chase time − p50 detected at pulse time) × 1.74 (this value represents the net p50 generated during chase)/(p105 generated during pulse)] × 100. Percent of degradation was calculated with the following equation: {[p105 generated during pulse − p105 remaining after chase − (net p50 generated during chase × 1.74)]/p105 generated during pulse} × 100. Multiplication of the radioactivity in p50 by 1.74 was done in order to correct for the ratio of cysteine and methionine residues between p105 and p50. (D) Removal of p105 lysine residues 1 to 30 completely inhibits IKKβ-induced degradation, while processing remains unaffected. COS-7 cells were transiently transfected for 24 h with cDNAs coding for p105-WT (containing lysine residues 1 [425] to 30 [967]; lanes 1 to 4), p105-Δ429-654/ΔK2-17 (lacking lysine residues 2 to 17; lanes 5 to 8), or p105-Δ429-654/ΔK2-17;K1,18-30R (lacking lysine residues 1 to 30; lanes 9 to 12). Where indicated, cDNA coding for constitutive active IKKβ was cotransfected. Processing and degradation of the different p105 mutants was monitored in a pulse-chase labeling and immunoprecipitation experiment as described in Materials and Methods and in the legend to Fig. 4B. Quantified data are also presented (for quantification, see the legend to panel C).

DISCUSSION

Generation of the p50 and p52 subunits of the NF-κB transcriptional activators from their respective precursors p105 and p100 are rare events in which the ubiquitin system is involved in limited processing rather than in complete destruction of its target substrates. Adding to the rarity is the finding that processing occurs both under basal conditions and following signaling, and it appears to be mediated by different ubiquitin system enzymes and signaling complexes. It is not surprising, therefore, that the mechanistic details of this complex process are still largely obscure. Lin and colleagues suggested that processing may occur cotranslationally, and synthesis of the

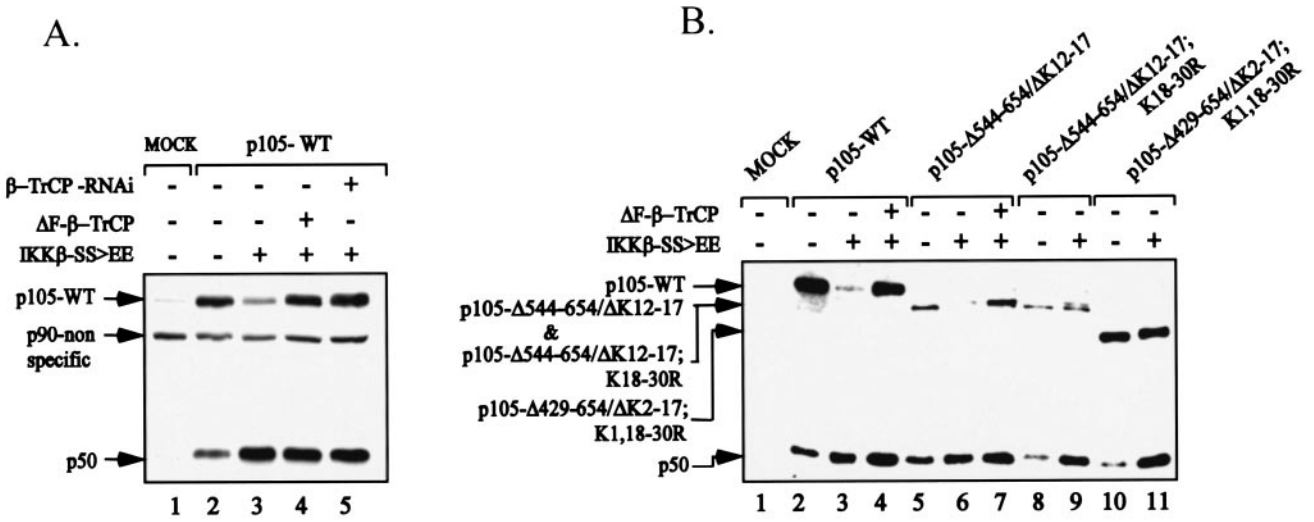

FIG. 6. β-TrCP is essential for stimulated degradation but not for stimulated processing of p105. (A) Suppression of β-TrCP inhibits degradation but not processing of p105. HEK 293 cells were transfected by either an empty vector (lane 1) or with cDNA coding for p105-WT (lanes 2 to 5). cDNAs coding for IKKβ (lanes 3 to 5) and ΔF-box β-TrCP (lane 4) or oligonucleotide for siRNA silencing of β-TrCP1 and -2 (lane 5) were transfected as described in Materials and Methods. Forty-eight hours after transfection with siRNA and 24 h after transfection with all other cDNAs, cells were harvested with RIPA buffer. Extracts representing an equal number of cells were resolved by SDS–10% PAGE and were blotted onto nitrocellulose paper, and proteins were visualized by using anti-p50 antibody and ECL as described in Materials and Methods. (B) Utilization of a dominant-negative ΔF-box β-TrCP or of p105 mutants that lack lysine residues 12 to 30 or 1 to 30 inhibits IKK-induced degradation but does not affect IKK-stimulated processing of p105. HEK 293 cells were transfected with either an empty vector (lane 1) or with cDNAs coding for p105-WT (lanes 2 to 4), p105-Δ544-654/ΔK12-17 (lanes 5 to 7), p105-Δ544-654/ΔK12-17;K18-30R (lanes 8 and 9), or p105-Δ429-654/ΔK2-17;K1,18-30R (lanes 10 and 11). Where indicated, cDNAs coding for ΔF-box β-TrCP and/or constitutively active IKKβ were cotransfected. Twenty-four hours after transfection cells were harvested with RIPA buffer, and disappearance of p105 and generation of p50 were monitored by using Western blot analysis as described in Materials and Methods and the legend to Fig. 4A. The anti-p50 antibody used here was different from the one that was used in the other experiments in that it did not recognize the 90-kDa nonspecific immune-reactive protein (marked once in panel A). This band masks the low-molecular-mass p105 deletion mutants.

entire precursor molecule p105 or p100 is not required for generation of p50 or p52, respectively (18, 22). Cotranslational processing occurs prior to the biosynthesis of the IKKβ phosphorylation and β-TrCP binding site in the C-terminal domain of the p105 molecule. Thus, such processing, if it occurs, must be constitutive. In contrast, several studies have shown increased, signal-induced formation of p50, though the extent of stimulation has been variable in the different experimental systems (6, 10, 24–26, 28). Heissmeyer and colleagues (14, 15) have shown that stimulation by IKKβ with subsequent ubiquitination by β-TrCP results in almost complete degradation of p105, while the increased generation of p50 is slight and inconsistent. These researchers argue that p50 is generated, most probably, from p105 in a constitutive, stimulation-independent manner. Orian and colleagues have shown that IKKβ and β-TrCP stimulate both degradation of p105 as well as increased formation of p50 (29). Taken together, these data suggest that p105 can undergo two distinct processes, degradation and limited processing, that are probably catalyzed and regulated independently. Thus, it is important to dissect the underlying mechanism(s) and role of each of the playing partners in these two processes. For example, it is possible that while IKKβ phosphorylates Ser residues necessary for recognition by β-TrCP, it also plays a ligase-independent role(s) in p105 metabolism. We used several experimental approaches to independently dissect the role of IKKβ and β-TrCP in p105 fate and p50 generation and to examine the possible existence of additional components in the system.

Initially we attempted to identify, via systematic mutagenesis, the lysine residue(s) targeted by β-TrCP and to examine whether inactivation of ubiquitination affects degradation of phosphorylated p105, its processing to p50, or both. As is seen in Fig. 3 and 5A, all 30 lysine residues in the IkBγ domain of p105 appear to be involved in ubiquitination of the molecule; gradual removal of these residues leads to a parallel and concomitant inhibition of β-TrCP-mediated ubiquitination, and there is clearly not a single lysine residue on which ubiquitination is completely dependent. Examination of the different mutant proteins (Fig. 4 and 5B to D) reveals that it takes the replacement of more than 13 lysine residues in the C-terminal domain of p105 in order to display a distinct and novel phenotype in which processing is maintained while degradation is gradually inhibited. Thus, as can be seen in Fig. 4, replacement of lysine residues 18 to 30 does not affect processing or degradation of p105. In contrast, as can be seen in Fig. 5C, the degradation of p105 that lacks lysine residues 12 to 30 is dramatically inhibited (88%; 3% versus 27%; compare lane 4 to lane 8), while the generation of p50 is almost not affected (50% versus 46%; compare lane 4 to lane 8 [see below]; all results were calculated based on the processing of p105 that lacks lysine residues 12 to 17). Similarly, as is seen in Fig. 5D, replacement of all 30 lysine residues in IκBγ eliminates degradation altogether without affecting processing. Importantly,

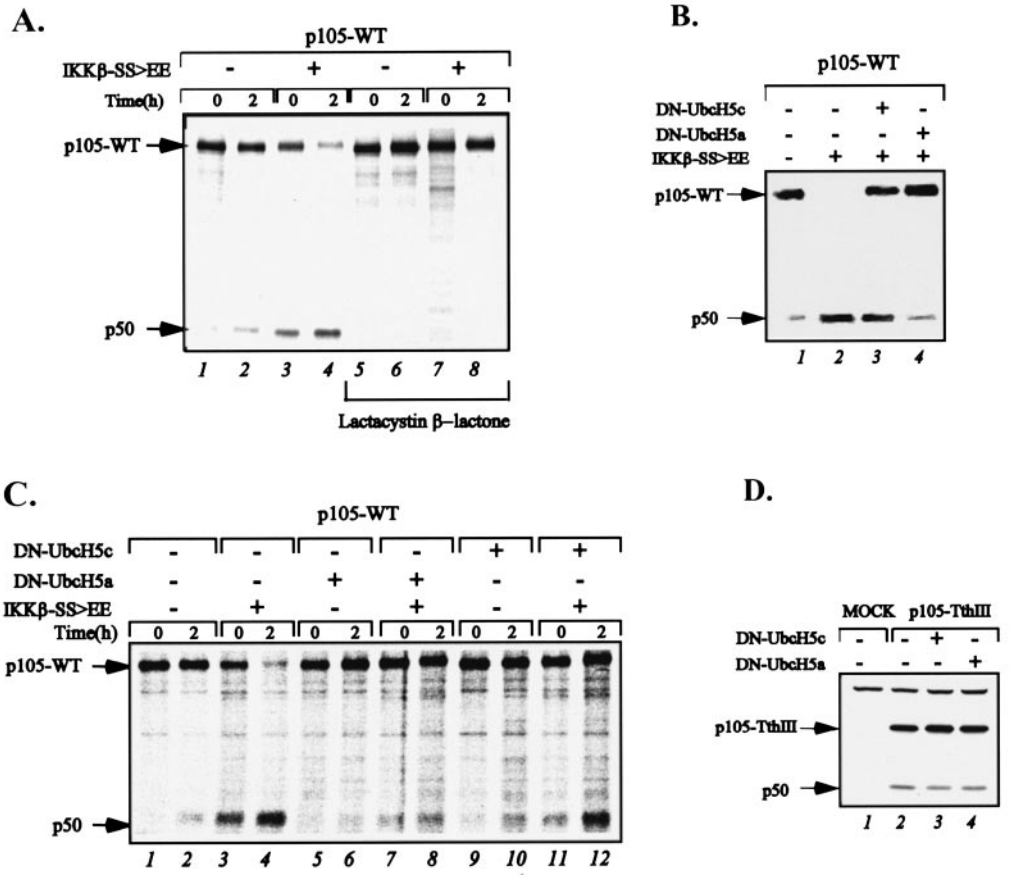

FIG. 7. Signal-induced processing and degradation of p105 are dependent on an active ubiquitin-proteasome pathway. (A) Inhibition of the proteasome suppresses signal-induced processing and degradation of p105. COS-7 cells were transiently transfected for 24 h with a cDNA coding for p105-WT. Where indicated, cDNA coding for constitutive active IKKβ was cotransfected. Processing and degradation of p105 were monitored in a pulse-chase labeling and immunoprecipitation experiment in the absence (lanes 1 to 4) or presence (lanes 5 to 8) of lactacystin β-lactone, a 20S proteasome inhibitor, as described in Materials and Methods and in the legend to Fig. 4B. (B) The ubiquitin-conjugating enzymes UbcH5c and UbcH5a act differently on IKKβ-mediated processing of p105. COS-7 cells were transiently transfected with a cDNA coding for p105-WT. Where indicated, cDNAs coding for constitutively active IKKβ, DN-UbcH5a, or DN-UbcH5c were cotransfected. Twenty-four hours after transfection cells were harvested, and disappearance of p105 and generation of p50 were monitored by using Western blot analysis as described in Materials and Methods and in the legend to Fig. 4A. (C) The ubiquitin-conjugating enzymes UbcH5C and UbcH5A act differently on IKKβ-mediated processing of p105. This experiment is similar to the one described in Fig. 5B, except that it was carried out by using pulse-chase labeling and immunoprecipitation of p105 and p50 as described in Materials and Methods and in the legend to Fig. 4B. (D) Neither UbcH5c nor UbcH5a E2s are required for basal processing of p105. COS-7 cells were transiently transfected by either an empty vector (lane 1) or with cDNAs coding for p105-TthIII 1 (p105 to 544) (lanes 2 to 4). Where indicated, a cDNA coding for DN-UbcH5c (lane 3) or DN-UbcH5a (lane 4) was cotransfected. Twenty-four hours after transfection cells were harvested, and levels of p105 and p50 were monitored by using Western blot analysis as described in Materials and Methods and in the legend to Fig. 4A.

the two processes still require IKK, as deletion of the IKK phosphorylation site in p105 eliminates both (Fig. 4B, lanes 9 to 12). Thus, by using systematic mutagenesis of lysine residues in the IκBγ domain of p105 we were able to demonstrate that IKK-dependent processing and degradation occur via two distinct mechanisms.

At that point it was important to dissect the role of β-TrCP in the two processes: is it involved in processing, degradation, or both? To study the role of the p105 ligase in the two processes, we utilized both specific siRNA to silence β-TrCP1 and β-TrCP2 mRNAs as well as expression of dominant-negative β-TrCP that lacks the F-box domain. As can be seen in Fig. 6, both elimination of β-TrCP expression (panel A) and sequestration of the substrate into an inactive β-TrCP E3 complex (panels A and B) completely inhibit degradation without affecting processing. It should be noted that in the case of p100, β-TrCP induces processing and not degradation (8), though a detailed analysis of this process has not been done. As β-TrCP is not involved in signal-induced and IKK-dependent p105 processing, it was important to show whether this process is at all dependent on the activity of the ubiquitin-proteasome system. It was known that the proteasome is involved in basal processing of p105 (31, 32). As can be seen in Fig. 7A, inhibition of the proteasome eliminates both IKK-dependent processing and degradation of p105. An important finding is that the process requires not only the proteasome but also the ubiquitination machinery. The E2 enzyme UbcH5c selectively inhibits only IKK-stimulated degradation of p105, while its homologue UbcH5a inhibits both (Fig. 7B and C). It is possible that UbcH5a acts with two different ligases, β-TrCP, which

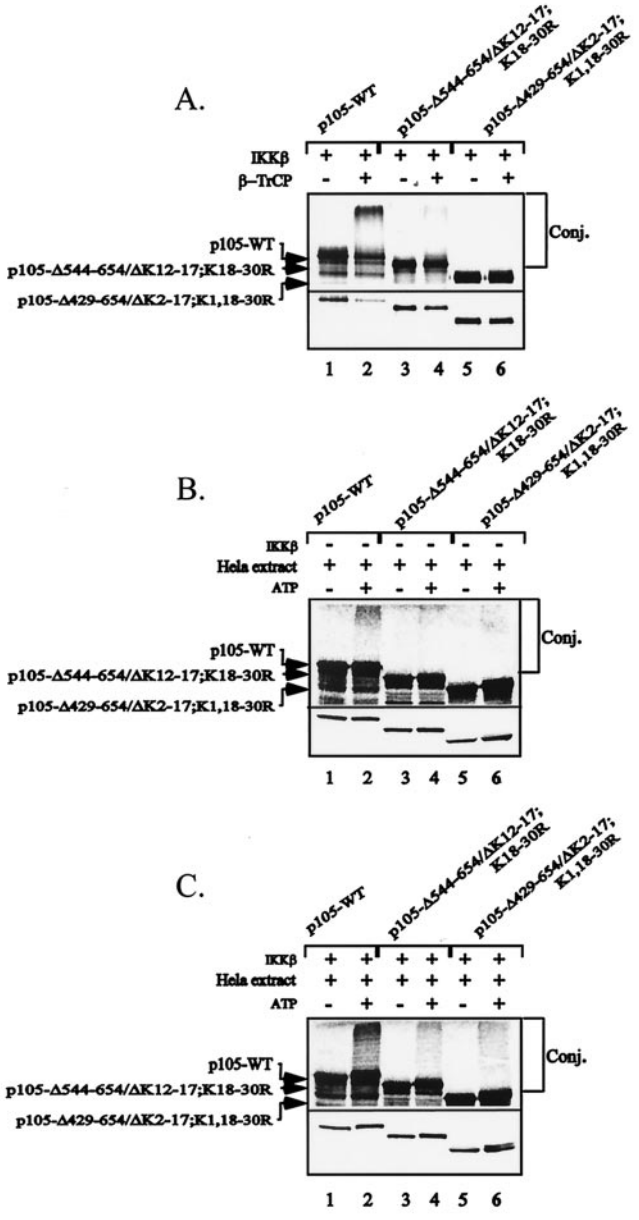

FIG. 8. IKKβ-phosphorylated p105 mutant that lacks all 30 lysine residues in its IκBγ domain is ubiquitinated by a non-TrCP ubiquitin ligase. (A) In vitro-translated, [^{35}S]methionine-labeled, and IKKβ-phosphorylated WT-p105 as well as p105 species that lack lysine residues 12 to 30 (p105-Δ544-654/ΔK12-17;K18-30R) and 1 to 30 (p105-Δ429-654/ΔK2-17;K1,18-30R) were subjected to SCF$^{\beta\text{-TrCP}}$-mediated conjugation in a cell-free system as described in Materials and Methods and in the legends to Fig. 2 and 3. (B) An experiment similar to the one described in panel A was carried out in crude HeLa extract rather then in a cell-free reconstituted system. Conjugation was monitored in the absence and presence of ATP. (C) An experiment the same as the one described in panel B was carried out, except that the [^{35}S]methionine-labeled p105s were phosphorylated by IKK prior to their incubation in the HeLa extract. Proteins were resolved by SDS–10% PAGE and were exposed to a PhosphorImager screen as described in Materials and Methods and in the legends to Fig. 2 and 3. The lower panels show the different p105 species as observed under reduced exposure to demonstrate that equal amounts of radioactive proteins were loaded on the gels. Conj., conjugates.

mediates degradation, and a yet-unknown ligase involved in processing. UbcH5c, on the other hand, acts only with the β-TrCP that mediates degradation, which was described earlier (11). It should be noted that equal amounts of the two E2 enzymes were expressed in the cells. Here, too, the IKK phosphorylation site is necessary for the E2 enzymes to mediate their reaction; processing of a truncated species of p105 (5) that lacks the IκBγ domain is not affected by either UbcH5a or UbcH5c. This basal processing, if at all mediated by the ubiquitin system, appears to be catalyzed by yet another E2.

An important question involves the identity of the E3 that mediates signal-induced processing. As is seen in Fig. 8A and as expected, β-TrCP does not conjugate p105 in which lysine residues 1 to 30 were replaced. In contrast, a crude HeLa cell extract does catalyze conjugation of the IκBγ lysine-less molecule, and conjugation is dependent on IKK (Fig. 8B and C). Thus, it appears that HeLa extract contains a ubiquitin ligase that requires IKK activity and is involved in processing, though not necessarily in a direct manner. This putative ligase does not bind to the IKK phosphorylation site, as binding of ΔF-box β-TrCP to the site does not eliminate processing (Fig. 6). IKK phosphorylation probably confers an alteration in the structure of the p105 molecule that allows ubiquitination by this putative E3 on lysine residues that reside in the p50 domain. Interestingly, this is also a rare case of conjugation on the nonleaving or nondegradable portion of a target substrate. It should be noted that p105 and p100 are conjugated by an additional, unidentified E3 that catalyzes conjugation under basal conditions and is not dependent on signaling (8, 30) (Fig. 7D).

An important problem is related to the requirement for three distinct ligases for processing and degradation of p105. Evolutionarily it makes sense to evolve two distinct mechanisms that will be involved in processing that occurs under basal conditions and following stimulation. The first process will provide the cell with the amount of p50 required for maintenance of the different cellular processes supported by NF-κB under basal conditions. Stress, such as that which occurs following apoptotic or inflammatory stimuli, requires instantaneous recruitment of large amounts of p50 and p65. This may come from the storage of subunit molecules that are anchored to cytosolic p105 and are released following stimulation-mediated processing and degradation of the p105-anchoring molecule (5, 13, 33, 36). Yet it is not immediately clear why nature has evolved two distinct mechanisms for stimulation-mediated processing and degradation. One possibility is that degradation occurs only when the p105 molecule is saturated with anchored p50 subunits which are then released, while processing is reserved for p105 molecules to which subunits have not been anchored, such as newly synthesized precursors. In this case, processing of a single p105 precursor will provide the cell with an additional p50 molecule. As for stimulation-induced degradation, it is possible that the p50-saturated p105 is not accessible to the enzymes involved in processing and had therefore required the evolvement of a new and distinct mechanism for release of the anchored subunits that involves a concomitant destruction of the carrier. An alternative explanation is that while degradation of the entire p105 molecule may provide a variety of anchored NF-κB subunits, processing will provide solely p50. These two different pathways may serve to generate distinct NF-κB species re-

quired for different cellular processes. For example, it has been shown that homodimeric p50 NF-κB is required for induction of lipopolysaccharide tolerance (3).

ACKNOWLEDGMENTS

This research was supported by grants from CapCure Israel—Centers of Excellence Program, the Israel Science Foundation founded by the Israeli Academy of Sciences and Humanities—Centers of Excellence Program and the German-Israeli Project Cooperation (DIP). A.C. is an Israel Cancer Research Fund professor. Infrastructural equipment was purchased with support of the Wolfson Charity Fund Center of Excellence for Studies on Turnover of Cellular Proteins and Its Implications to Human Diseases. S.C. is supported by the B. de Rotschild Fund for the Advancement of Science in Israel.

REFERENCES

1. **Alkalay, I., A. Yaron, A. Hatzubai, S. Jung, A. Avraham, O. Gerlitz, I. Pashut-Lavon, and Y. Ben-Neriah.** 1995. In vivo stimulation of IκB phosphorylation is not sufficient to activate NF-κB. Mol. Cell. Biol. **15:**1294–1301.
2. **Baldi, L., K. Brown, G. Franzoso, and U. Siebenlist.** 1996. Critical role for lysines 21 and 22 in signal-induced, ubiquitin-mediated proteolysis of IκBα. J. Biol. Chem. **271:**376–379.
3. **Bohuslav, J., V. V. Kravchenko, G. C. Parry, J. H. Erlich, S. Gerondakis, N. Mackman, and R. J. Ulevitch.** 1998. Regulation of an essential innate immune response by the p50 subunit of NF-κB. J. Clin. Investig. **102:**1645–1652.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Cohen, S., A. Orian, and A. Ciechanover.** 2001. Processing of p105 is inhibited by docking of p50 active subunits to the ankyrin repeat domain, and inhibition is alleviated by signaling via the carboxyl-terminal phosphorylation/ubiquitin-ligase binding domain. J. Biol. Chem. **276:**26769–26776.
6. **Donald, R., D. W. Ballard, and J. Hawiger.** 1995. Proteolytic processing of NF-κB/IκB in human monocytes. ATP-dependent induction by pro-inflammatory mediators. J. Biol. Chem. **270:**9–12.
7. **Fan, C. M., and T. Maniatis.** 1991. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. Nature **354:**395–398.
8. **Fong, A., and S.-C. Sun.** 2002. Genetic evidence for the essential role of β-transducin repeat-containing protein in the inducible processing of NF-κB2/p100. J. Biol. Chem. **277:**22111–22114.
9. **Foo, S. Y., and G. P. Nolan.** 1999. NF-κB to the rescue: RELs, apoptosis and cellular transformation. Trends Genet. **15:**229–235.
10. **Fujimoto, K., H. Yasuda, Y. Sato, and K. Yamamoto.** 1995. A role for phosphorylation in the proteolytic processing of the human NF-κB1 precursor. Gene **165:**183–189.
11. **Gonen, H., B. Bercovich, A. Orian, A. Carrano, C. Takizawa, K. Yamanaka, M. Pagano, K. Iwai, and A. Ciechanover.** 1999. Identification of the ubiquitin carrier proteins, E2s, involved in signal-induced conjugation and subsequent degradation of IκBα. J. Biol. Chem. **274:**14823–14830.
12. **Harhaj, E. W., S. B. Maggirwar, and S. C. Sun.** 1996. Inhibition of p105 processing by NF-κB proteins in transiently transfected cells. Oncogene **12:**2385–2392.
13. **Hatada, E. N., A. Nieters, F. G. Wulczyn, M. Naumann, R. Meyer, G. Nucifora, T. W. McKeithan, and C. Scheidereit.** 1992. The ankyrin repeat domains of the NF-κB precursor p105 and the protooncogene bcl-3 act as specific inhibitors of NF-κB DNA binding. Proc. Natl. Acad. Sci. USA **89:**2489–2493.
14. **Heissmeyer, V., D. Krappmann, E. N. Hatada, and C. Scheidereit.** 2001. Shared pathways of IκB kinase-induced SCF(βTrCP)-mediated ubiquitination and degradation for the NF-κB precursor p105 and IκBα. Mol. Cell. Biol. **21:**1024–1035.
15. **Heissmeyer, V., D. Krappmann, F. G. Wulczyn, and C. Scheidereit.** 1999. NF-κB p105 is a target of IκB kinases and controls signal induction of Bcl-3-p50 complexes. EMBO J. **18:**4766–4778.
16. **Hershko, A., and H. Heller.** 1985. Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates. Biochem. Biophys. Res. Commun. **128:**1079–1086.
17. **Hershko, A., and I. A. Rose.** 1987. Ubiquitin-aldehyde: a general inhibitor of ubiquitin-recycling processes. Proc. Natl. Acad. Sci. USA **84:**1829–1833.
18. **Heusch, M., L. Lin, R. Geleziunas, and W. C. Greene.** 1999. The generation of nfkb2 p52: mechanism and efficiency. Oncogene **18:**6201–6208.
19. **Karin, M., and Y. Ben-Neriah.** 2000. Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu. Rev. Immunol. **18:**621–663.
20. **Kawakami, T., T. Chiba, T. Suzuki, K. Iwai, K. Yamanaka, N. Minato, H. Suzuki, N. Shimbara, Y. Hidaka, F. Osaka, M. Omata, and K. Tanaka.** 2001. NEDD8 recruits E2-ubiquitin to SCF E3 ligase. EMBO J. **20:**4003–4012.
21. **Lee, C., M. P. Schwartz, S. Prakash, M. Iwakura, and A. Matouschek.** 2001. ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. Mol. Cell. **7:**627–637.
22. **Lin, L., G. N. DeMartino, and W. C. Greene.** 1998. Cotranslational biogenesis of NF-κB p50 by the 26S proteasome. Cell **92:**819–828.
23. **Lin, L., and S. Ghosh.** 1996. A glycine-rich region in NF-κB p105 functions as a processing signal for the generation of the p50 subunit. Mol. Cell. Biol. **16:**2248–2254.
24. **MacKichan, M. L., F. Logeat, and A. Israël.** 1996. Phosphorylation of p105 PEST sequence via a redox-insensitive pathway up-regulates processing of p50 NF-κB. J. Biol. Chem. **271:**6084–6091.
25. **Mellits, K. H., R. T. Hay, and S. Goodbourn.** 1993. Proteolytic degradation of MAD3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. Nucleic Acids Res. **21:**5059–5066.
26. **Mercurio, F., J. A. DiDonato, C. Rosette, and M. Karin.** 1993. p105 and p98 precursor proteins play an active role in NF-κB-mediated signal transduction. Genes Dev. **7:**705–718.
27. **Mercurio, F., B. W. Murray, A. Shevchenko, B. L. Bennett, D. B. Young, J. W. Li, G. Pascual, A. Motiwala, H. Zhu, M. Mann, and A. M. Manning.** 1999. IκB Kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. Mol. Cell. Biol. **19:**1526–1538.
28. **Naumann, M., and C. Scheidereit.** 1994. Activation of NF-κB in vivo is regulated by multiple phosphorylations. EMBO J. **13:**4597–4607.
29. **Orian, A., H. Gonen, B. Bercovich, I. Fajerman, E. Eytan, A. Israël, F. Mercurio, K. Iwai, A. L. Schwartz, and A. Ciechanover.** 2000. SCF(β-TrCP) ubiquitin ligase-mediated processing of NF-κB p105 requires phosphorylation of its C-terminus by IκB kinase. EMBO J. **19:**2580–2591.
30. **Orian, A., A. L. Schwartz, A. Israël, S. Whiteside, C. Kahana, and A. Ciechanover.** 1999. Structural motifs involved in ubiquitin-mediated processing of the NF-κB precursor p105: roles of the glycine-rich region and a downstream ubiquitination domain. Mol. Cell. Biol. **19:**3664–3673.
31. **Orian, A., S. Whiteside, A. Israël, I. Stancovski, A. L. Schwartz, and A. Ciechanover.** 1995. Ubiquitin-mediated processing of NF-κB transcriptional activator precursor p105. Reconstitution of a cell-free system and identification of the ubiquitin-carrier protein, E2, and a novel ubiquitin-protein ligase, E3, involved in conjugation. J. Biol. Chem. **270:**21707–21714.
32. **Palombella, V. J., O. J. Rando, A. L. Goldberg, and T. Maniatis.** 1994. The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell **78:**773–785.
33. **Rice, N. R., M. L. MacKichan, and A. Israël.** 1992. The precursor of NF-κB p50 has IκB-like functions. Cell **71:**243–253.
34. **Scherer, D. C., J. A. Brockman, Z. Chen, T. Maniatis, and D. W. Ballard.** 1995. Signal-induced degradation of IκBα requires site-specific ubiquitination. Proc. Natl. Acad. Sci. USA **92:**11259–11263.
35. **Senftleben, U., Y. Cao, G. Xiao, F. R. Greten, G. Krahn, G. Bonizzi, Y. Chen, Y. Hu, A. Fong, S.-C. Sun, and M. Karin.** 2001. Activation by IKKα of a second, evolutionary conserved, NF-κB signaling pathway. Science **293:**1495–1499.
36. **Sun, S. C., P. A. Ganchi, C. Beraud, D. W. Ballard, and W. C. Greene.** 1994. Autoregulation of the NF-κB transactivator RelA (p65) by multiple cytoplasmic inhibitors containing ankyrin motifs. Proc. Natl. Acad. Sci. USA **91:**1346–1350.