

## $\beta$ TrCP-Mediated Proteolysis of NF- $\kappa$ B1 p105 Requires Phosphorylation of p105 Serines 927 and 932

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Received 23 September 2002/Accepted 27 September 2002

**NF- $\kappa$ B1 p105 functions both as a precursor of NF- $\kappa$ B1 p50 and as a cytoplasmic inhibitor of NF- $\kappa$ B. Following the stimulation of cells with tumor necrosis factor alpha (TNF- $\alpha$ ), the I $\kappa$ B kinase (IKK) complex rapidly phosphorylates NF- $\kappa$ B1 p105 on serine 927 in the PEST region. This phosphorylation is essential for TNF- $\alpha$  to trigger p105 degradation, which releases the associated Rel/NF- $\kappa$ B subunits to translocate into the nucleus and regulate target gene transcription. Serine 927 resides in a conserved motif (Asp-Ser<sup>927</sup>-Gly-Val-Glu-Thr-Ser<sup>932</sup>) homologous to the IKK target sequence in I $\kappa$ B $\alpha$ . In this study, TNF- $\alpha$ -induced p105 proteolysis was revealed to additionally require the phosphorylation of serine 932. Experiments with IKK1<sup>-/-</sup> and IKK2<sup>-/-</sup> double knockout embryonic fibroblasts demonstrate that the IKK complex is essential for TNF- $\alpha$  to stimulate phosphorylation on p105 serines 927 and 932. Furthermore, purified IKK1 and IKK2 can each phosphorylate a glutathione *S*-transferase-p105<sub>758-967</sub> fusion protein on both regulatory serines in vitro. IKK-mediated p105 phosphorylation generates a binding site for  $\beta$ TrCP, the receptor subunit of an SCF-type ubiquitin E3 ligase, and depletion of  $\beta$ TrCP by RNA interference blocks TNF- $\alpha$ -induced p105 ubiquitination and proteolysis. Phosphopeptide competition experiments indicate that  $\beta$ TrCP binds p105 more effectively when both serines 927 and 932 are phosphorylated. Interestingly, however,  $\beta$ TrCP affinity for the IKK-phosphorylated sequence on p105 is substantially lower than that on I $\kappa$ B $\alpha$ . Thus, it appears that reduced p105 recruitment of  $\beta$ TrCP and subsequent ubiquitination may contribute to delayed p105 proteolysis after TNF- $\alpha$  stimulation relative to that for I $\kappa$ B $\alpha$ .**

NF- $\kappa$ B transcription factors play an important role in the regulation of genes involved in immune and inflammatory responses, apoptosis, and development (7, 11). Mammals express five NF- $\kappa$ B proteins that bind DNA as homo- and heterodimers: RelA, RelB, c-Rel, NF- $\kappa$ B1 p50, and NF- $\kappa$ B2 p52 (22). NF- $\kappa$ B1 and NF- $\kappa$ B2 are synthesized as large precursors of 105 kDa (p105) and 100 kDa (p100), respectively, that require proteolytic processing by the proteasome to produce their respective p50 and p52 NF- $\kappa$ B subunits (1, 22).

Mature NF- $\kappa$ B dimers are retained in the cytoplasm of unstimulated cells through their interaction with a family of inhibitory proteins, termed I $\kappa$ Bs (7). A number of agonists activate NF- $\kappa$ B, including proinflammatory cytokines, lipopolysaccharide, double-stranded RNA, and the viral transactivator Tax. These stimuli induce I $\kappa$ B phosphorylation via the I $\kappa$ B kinase (IKK) complex, which contains two related kinases, IKK1 (IKK $\alpha$ ) and IKK2 (IKK $\beta$ ), and a structural subunit, NEMO (IKK $\gamma$ ). This triggers I $\kappa$ B ubiquitination and subsequent proteolysis by the proteasome (11). NF- $\kappa$ B dimers are thereby released to translocate into the nucleus, where their transcriptional activity is also regulated by phosphorylation (7).

The NF- $\kappa$ B1 precursor p105 functions as an I $\kappa$ B due to the presence of ankyrin repeats in its C terminus and retains as-

sociated p50, c-Rel, and RelA in the cytoplasm (16, 20). Two proteolytic pathways control cellular levels of p105: limited (processing to p50) and complete (degradation) (11). Following the stimulation of cells with proinflammatory cytokines, such as tumor necrosis factor alpha (TNF- $\alpha$ ) or interleukin-1 (IL-1), p105 is phosphorylated and then more rapidly proteolyzed by the proteasome (14–16). This predominantly results in accelerated p105 degradation, rather than increased processing to p50, which is largely a constitutive process (9, 24). Freed Rel subunits translocate into the nucleus to modulate target gene transcription. Analysis of knockout mice which lack the C-terminal (I $\kappa$ B-like) half of p105 while still expressing p50 has revealed an essential role for p105 in the proper regulation of p50 homodimers, which is required for the correct control of inflammatory responses (10).

The C-terminal PEST domain of p105 contains a conserved motif (Asp-Ser<sup>927</sup>-Gly-Val-Glu-Thr-Ser<sup>932</sup>) which is homologous to the IKK target sequence in I $\kappa$ B $\alpha$  (Fig. 1A) (8, 21). Our laboratory has recently demonstrated that phosphorylation of serine 927 is essential for the signal-induced degradation of p105 triggered by either IKK2 overexpression or stimulation of cells with TNF- $\alpha$  (21). Mutation of serine 932 to alanine was also found to reduce the rate of proteolysis triggered by IKK2 overexpression. In the present study, the role of serine 932 in signal-induced degradation was investigated in more detail. These experiments reveal that the phosphorylation of both serine 927 and serine 932 by the IKK complex is essential for TNF- $\alpha$  to trigger p105 ubiquitination and proteolysis.

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## MATERIALS AND METHODS

**cDNA constructs and antibodies.** cDNAs encoding hemagglutinin (HA) epitope-tagged p105 and serine-to-alanine point mutant HA-p105 derivatives were described in an earlier study (21). For transient transfection experiments, these were subcloned into the pcDNA3 expression vector (Invitrogen Corp.), whereas the pMX-1 expression vector (Ingenius) was used to generate stably transfected HeLa cell lines. Expression vectors encoding Flag-epitope-tagged IKK2 (FL-IKK2), βTrCP1 (FL-βTrCP1), and βTrCP2 (FL-βTrCP2) have been described previously (17, 23).

HA-p105 was immunoprecipitated using 12CA5 monoclonal antibody (MAb) and detected in Western blots with a commercial anti-HA MAb (3F10; Roche Molecular Biochemicals). Endogenous p105 was immunoprecipitated and detected in Western blots by using the anti-p105C antibody, which recognizes the C terminus of human p105 (21). The anti-phospho-S<sup>927</sup>-p105 antibody has been described in previous work (21). To generate the anti-phospho-S<sup>932</sup>-p105 antibody, a peptide was synthesized corresponding to residues 922 through 935 of human p105 in which serine 932 was phosphorylated. After high-pressure liquid chromatography purification, this phosphopeptide was coupled to keyhole limpet hemocyanin (Pierce) and injected into rabbits. For use in Western blots, immunoglobulin G was isolated from 4 ml of antiserum by using protein G-Sepharose (Amersham Bioscience) and then affinity purified on a column of Affi-Gel 10 (Bio-Rad) coupled to 10 mg of phosphopeptide after first passing through an Affi-Gel 10 column coupled to 10 mg of unphosphorylated peptide from residues 922 to 935. Antibodies recognizing IκBα (sc-371; Santa Cruz), βTrCP1 and βTrCP2 (sc-8863; Santa Cruz), and the Flag epitope (M2 MAb; Sigma) were purchased from the indicated suppliers. Anti-glutathione S-transferase (GST) MAb was kindly provided by Steve Dillworth (Hammersmith Hospital, London, United Kingdom).

**Cell culture and stable transfections.** HeLa cells (Ohio subline from the European Collection of Cell Cultures) and mouse embryonic fibroblasts (EFs) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp.) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (50 U/ml). For NIH 3T3 fibroblasts, 10% newborn calf serum was substituted for FCS. IKK1 and IKK2 double knockout (IKK1/2<sup>-/-</sup>) EFs were generously provided by Mark Schmitt and Inder Verma (The Salk Institute, San Diego, Calif.) (13).

The generation of stably transfected HeLa cells was carried out as described previously (21). Expression of HA-p105 was assessed by Western blotting.

**Protein analyses.** To analyze HA-p105 proteolysis in stably transfected HeLa clones, cells were plated at  $6 \times 10^5$  per well of a six-well plate (Nunc). After 18 h in culture, cells were washed with phosphate-buffered saline and cultured in methionine- and cysteine-free minimal essential Eagle's medium (Sigma) for 90 min. Cells were pulse-labeled with 2.65 MBq of [<sup>35</sup>S]methionine- [<sup>35</sup>S]cysteine (Pro-Mix; Amersham Bioscience) for 45 min and chased for the indicated times in complete medium (DMEM plus 2% FCS) alone (control) or complete medium supplemented with TNF-α (20 ng/ml; Amersham Bioscience). Cells were lysed in buffer A (1% NP-40, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, plus a cocktail of protease inhibitors [Roche]) supplemented with 0.1% sodium dodecyl sulfate (SDS) and 0.5% deoxycholate (RIPA buffer), and HA-p105 was immunoprecipitated as described previously (3). Labeled bands were quantified by laser densitometry (calibrated imaging densitometer; Bio-Rad). Pulse-chase experiments were carried out at least three times with similar results.

For ubiquitination experiments, HeLa cells were seeded at  $2 \times 10^6$  cells per 90-mm-diameter dish and left in culture until confluent (1 to 2 days). Cells were then treated with 50 μM N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) proteasome inhibitor (Sigma) for 1 h and incubated for a further 15 min in the presence of TNF-α or control medium. Cells were extracted in 1.2 ml of buffer A per dish. After centrifugation, HA-p105 was immunoprecipitated from lysates of four dishes, resolved on a SDS-6% polyacrylamide gel electrophoresis (PAGE) gel, and Western blotted.

**Analysis of p105 phosphorylation.** To analyze p105 phosphorylation on serines 927 and 932 in NIH 3T3 cells, HA-p105 was transiently expressed alone or together with FL-IKK2 by using Lipofectamine (Invitrogen Corp.). Cells were treated with 20 μM MG132 proteasome inhibitor (Biomol Research Labs) for the last 4 h of a 48-h culture and then lysed in buffer A. Phosphorylation was determined by immunoblotting of anti-HA immunoprecipitates with the indicated phosphopeptide-specific antibodies. Analysis of the phosphorylation of serines 927 and 932 on endogenous p105 in HeLa cells was carried out as described previously (21).

**In vitro kinase assays.** His<sub>6</sub>-IKK1 and His<sub>6</sub>-IKK2 were expressed in Sf9 insect cells by baculovirus infection and purified to near homogeneity as described

previously (21). One hundred nanograms of recombinant IKK protein was incubated at room temperature in 50 μl of kinase buffer (25 mM Tris [pH 7.5], 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium vanadate, 10 mM MgCl<sub>2</sub>) plus 20 μM ATP and 5 μg of purified GST-p105<sub>758-967</sub>, GST-p105<sub>758-967</sub>(S927A), or GST-p105<sub>758-967</sub>(S932A) protein. The reaction was stopped by the addition of 50 μl of 2× Laemmli sample buffer (12), and proteins were Western blotted with anti-phospho-Ser<sup>927</sup> or anti-phospho-Ser<sup>932</sup> antibody. Equal protein loading was confirmed by reprobing blots with an anti-GST MAb.

For in vitro phosphorylation experiments with endogenous IKK complex, control or TNF-α-stimulated HeLa cells ( $5 \times 10^6$  per 90-mm dish) were lysed in buffer A and immunoprecipitated by using anti-NEMO antibody (sc-8330; Santa Cruz). Immunoprecipitates were washed four times in buffer A and twice in kinase buffer. Kinase reactions were carried out as described above.

**RNA interference.** Depletion of endogenous βTrCP expression was achieved by RNA interference (6). Small interfering RNAs (siRNAs) for human βTrCP1 and βTrCP2 were synthesized by Xeragon Inc. The sequences of βTrCP siRNAs were GUGGAAUUUGUGGAACAUCCTT (sense) and GAUGUCCACAAA UCCACTT (antisense). At 12 to 16 h prior to transfection, 10<sup>5</sup> HeLa cells were seeded in six-well plates (Costar) in DMEM supplemented with 10% FCS. Cells were transfected with βTrCP siRNAs (0.4 nmol per well) by using Oligofectamine (Invitrogen Corp.) according to the manufacturer's instructions. After 72-h culture, cells were stimulated with TNF-α and then lysed in buffer A. Experiments with other synthetic siRNAs demonstrated that the effects of βTrCP siRNA treatment were specific by using IκBα degradation as a readout (data not shown).

Endogenous βTrCP1 and βTrCP2 were not detected on the Western blot of total cell lysates. Therefore, to determine the effectiveness of siRNAs in decreasing βTrCP1 and βTrCP2 levels, siRNAs or control transfected cells were lysed in buffer A (1 ml) and centrifuged lysates were incubated overnight with phospho-S32/S36 IκBα peptide (Fig. 1C) coupled to Affi-Gel 10 beads (Bio-Rad). Beads were then washed four times in buffer A and isolated endogenous βTrCP1 and βTrCP2 quantified by Western blotting. The same methodology was used to determine whether endogenous βTrCP1 and βTrCP2 could directly bind to p105-derived phosphopeptides (Fig. 1C).

**Peptides.** Synthetic p105 peptides (doubly, singly, and non-phosphorylated) were purchased from the University of Bristol Biochemistry Department, and IκBα peptides were purchased from SynPep (Dublin, Calif.). All peptides were purified by high-pressure liquid chromatography (>90%) and verified by mass spectrometry. The sequences of the peptides used are shown in Fig. 1C.

**βTrCP binding assays.** FL-βTrCP1 and FL-βTrCP2 were synthesized together from their respective expression vectors and labeled with [<sup>35</sup>S]methionine (Amersham Bioscience) by cell-free translation (25-μl reaction volume; Promega TNT-coupled rabbit reticulocyte system). GST-p105<sub>758-967</sub> fusion protein was phosphorylated with His<sub>6</sub>-IKK2 as described above, bound to glutathione-Sepharose beads (10 μl; Amersham Bioscience), and washed three times in buffer A. Translated proteins, diluted to 200 μl in buffer A, were then incubated with the phospho-GST-p105<sub>758-967</sub> beads overnight at 4°C with the indicated phosphopeptides or with no addition. Beads were washed a further four times in buffer A, and <sup>35</sup>S-labeled protein was visualized by autoradiography after SDS-8% PAGE.

To determine the association of βTrCP1 and βTrCP2 with phospho-p105 in vivo, HA-p105 HeLa cells were transiently transfected with vectors encoding FL-βTrCP1 and FL-βTrCP2 (0.5 μg each) by using Lipofectamine (Invitrogen Corp.). After 48-h culture, cells were stimulated with TNF-α for 15 min or left unstimulated. HA-p105 was then isolated from cell lysates by immunoprecipitation with anti-HA MAb, and Western blots were probed with anti-FL MAb to detect HA-p105-associated FL-βTrCP1 or FL-βTrCP2.

**In vitro IκBα ubiquitination.** [<sup>35</sup>S]Methionine-labeled IκBα was prepared as described previously (26) and then phosphorylated by recombinant, constitutively active IKK2 (IKK2-EE) (17). Immunopurified SCF<sup>βTrCP</sup> complex was preincubated with 5 to 650 μM concentrations of phosphorylated or unphosphorylated p105 or IκBα peptide (Fig. 1C) for 15 min and then mixed with phospho-IκBα, E1, and E2. Ubiquitination was performed as described previously (26), and ubiquitinated products were analyzed by SDS-PAGE (8% acrylamide) and subsequent autoradiography.

## RESULTS

**Serine 932 is essential for TNF-α-induced p105 degradation.** The Ley laboratory has previously demonstrated that a serine 932-to-alanine mutant of HA-p105 displays delayed proteolysis compared to wild-type HA-p105 when coexpressed

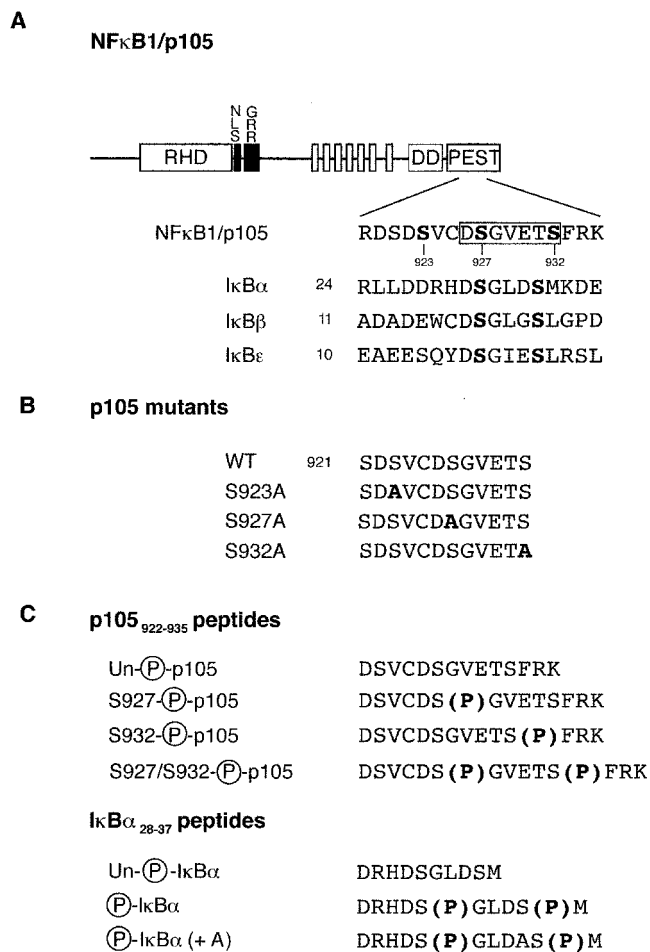


FIG. 1. IKK target sequences, p105 point mutants, and synthetic phosphopeptides. (A) Schematic representation of mammalian NF- $\kappa$ B1 p105. The relative positions of the Rel homology domain (RHD), nuclear localizing signal (NLS), glycine-rich region (GRR), ankyrin repeats (vertical rectangles), death domain (DD), and PEST domain are shown. An alignment of the IKK target sequence in the p105 PEST region with those in I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$  is also shown (all human proteins). (B) Serine-to-alanine point mutants of HA-p105. WT, wild type. (C) Sequences of synthetic p105 and I $\kappa$ B $\alpha$  peptides. P, phosphorylated residue.

with FL-IKK2 in NIH 3T3 cells. This suggested that the residue might play a regulatory role in signal-induced p105 proteolysis (21). Since the IKK complex is essential for cytokine-induced p105 degradation (21), it was important to determine whether serine 932 is important for p105 proteolysis triggered by cytokine stimulation of the endogenous IKK complex. To investigate this, HeLa cells were transfected with plasmids encoding HA-p105 or HA-p105(S932A) and stable clones were isolated after G418 selection (Fig. 1B). A pulse-chase metabolic labeling experiment was carried out with a representative clone that expressed levels of HA-p105(S932A) similar to that of a control clone expressing HA-p105. Stimulation with TNF- $\alpha$  increased the rate of turnover of HA-p105 as expected (Fig. 2A and B). In contrast, TNF- $\alpha$  stimulation consistently had no detectable effect on the rate of proteolysis of HA-p105(S932A). Analysis of the 90-min point confirmed that there was no significant difference in the amount of HA-p105(S932A)

remaining  $\pm$  TNF- $\alpha$  ( $P = 0.23$ ;  $n = 5$ ). However, the mobility of the HA-p105(S932A) band was retarded and became less distinct after TNF- $\alpha$  stimulation, probably due to phosphorylation on amino acids other than serine 932 (Fig. 2B). As reported previously (21), TNF- $\alpha$  stimulation did not increase the proteolysis of HA-p105(S927A) (Fig. 2A). Control experiments demonstrated that I $\kappa$ B $\alpha$  was rapidly and completely degraded following TNF- $\alpha$  stimulation in the HA-p105(S932A) clone similar to that in the wild-type HA-p105 clone, confirming that the TNF- $\alpha$  signaling pathway controlling the IKK complex was intact in the former cell line (Fig. 2C). Thus, serine 932 is essential for TNF- $\alpha$  to efficiently induce the proteolysis of HA-p105.

It was possible that the inhibitory effect of the S932A mutation on TNF- $\alpha$ -induced proteolysis of HA-p105 was due to the indirect effects on the phosphorylation of serine 927, which has previously been shown to be essential for the signal-induced degradation of p105 (8, 21). To investigate this possibility, HA-p105 was immunoprecipitated from the HA-p105(S932A) clone with or without TNF- $\alpha$  stimulation and Western blotted with anti-phospho-S<sup>927</sup>-p105 antibody. TNF- $\alpha$ -induced serine 927 phosphorylation of HA-p105(S932A) was similar to that of wild-type HA-p105 (Fig. 2D). Thus, the S932A mutation did not mediate its inhibitory effects on HA-p105 proteolysis by blocking the phosphorylation of serine 927.

A recent study demonstrated that recombinant IKK2 phosphorylated a p105<sub>917-968</sub> fusion protein on a residue equivalent to serine 923 in the full-length p105 protein (8). Furthermore, proteolysis of a serine 923-to-alanine point mutant of p105 was reduced when coexpressed in 293 cells with IKK2 and  $\beta$ TrCP compared to the wild-type protein. In contrast, previous experiments from this laboratory did not suggest an important role for serine 923 in degradation of HA-p105 triggered by FL-IKK2 overexpression in NIH 3T3 fibroblasts (21). To investigate whether serine 923 is required for the cytokine-induced degradation of p105, stable HeLa clones expressing HA-p105(S923A) were generated. Stimulation of a representative clone with TNF- $\alpha$  increased the rate of HA-p105(S923A) proteolysis to a degree similar to that for wild-type HA-p105 (Fig. 2A). Analysis of the 90-min chase time point confirmed that there was no significant difference in the amount of HA-p105(S923A) remaining (65%) compared to that of wild-type HA-p105 (60%) in TNF- $\alpha$ -stimulated cells ( $P = 0.372$ ;  $n = 5$ ). Thus, consistent with previous data (21), serine 923 is not required for stimulus-induced p105 degradation.

In conclusion, these data indicate an essential role for both serines 927 and 932 in the proteolysis of p105 triggered by TNF- $\alpha$  stimulation, whereas serine 923 is not required for TNF- $\alpha$ -induced p105 proteolysis.

**TNF- $\alpha$  induces the rapid phosphorylation of p105 on serine 932.** The previous experiments indicate an important role for serine 932 in p105 proteolysis triggered by stimulation with TNF- $\alpha$ . However, it was important to obtain direct evidence that serine 932 was actually phosphorylated in vivo to rule out the inhibitory effects on p105 proteolysis resulting from putative structural alterations caused by the S932A mutation. To investigate this, rabbits were immunized with a synthetic peptide corresponding to residues 922 to 935 of human p105 in which serine 932 was phosphorylated and specific antibody was purified on a phosphopeptide affinity column.

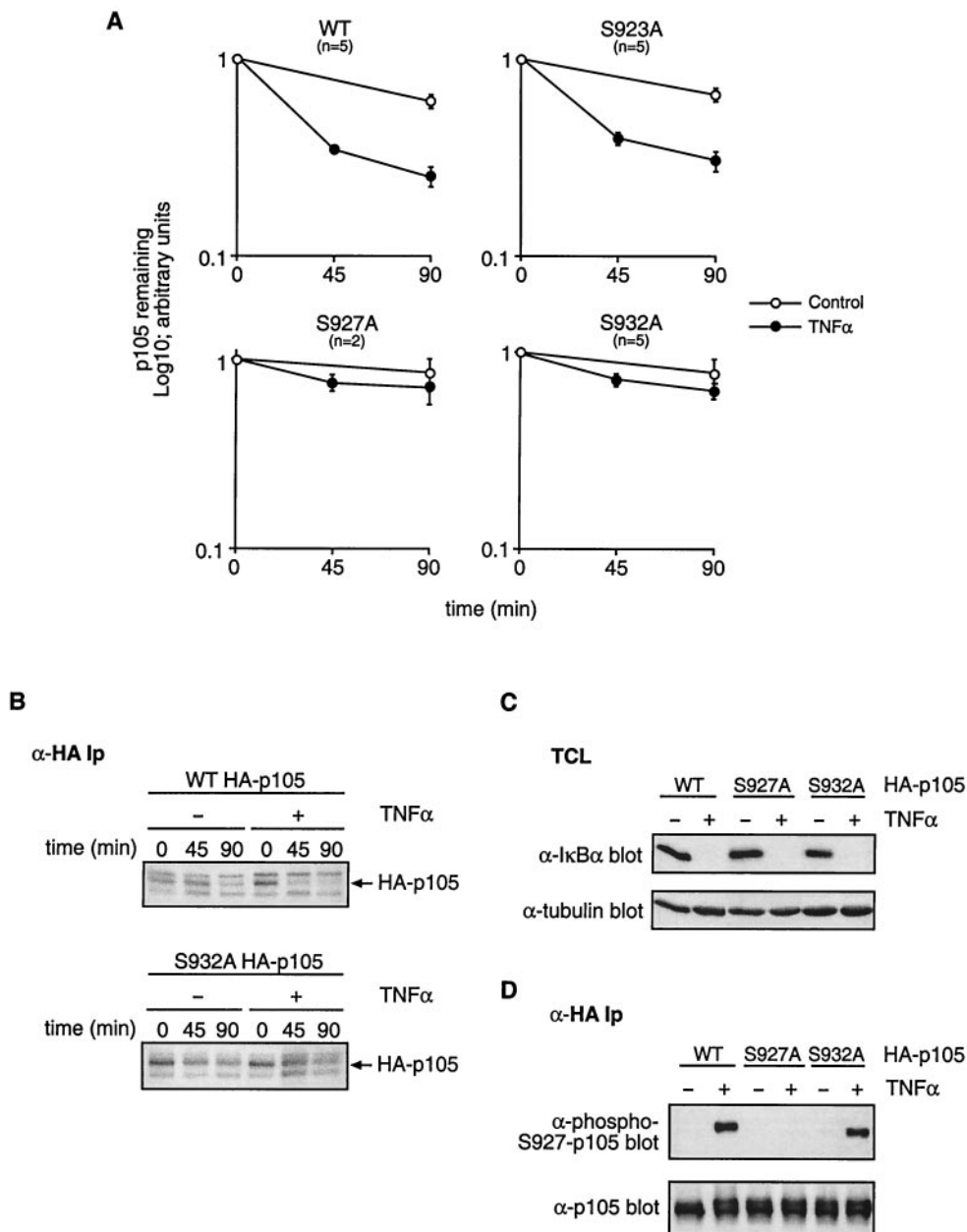


FIG. 2. Serine 932 is essential for TNF-α to trigger proteolysis of p105. (A) Clones of HeLa cells stably transfected with HA-p105, HA-p105 (S923A), HA-p105(S927A), or HA-p105(S932A) were metabolically pulse-labeled with [<sup>35</sup>S]methionine-[<sup>35</sup>S]cysteine (45 min) and then chased for the indicated times in complete medium (control) or complete medium supplemented with TNF-α. Anti-HA immunoprecipitates were resolved by SDS-7.5% PAGE and revealed by fluorography. Amounts of immunoprecipitated wild-type (WT) and point-mutated HA-p105 were quantified by laser densitometry (mean ± standard error of the mean; n = 5). (B) HeLa cells stably transfected with either HA-p105 or HA-p105(S932A) were analyzed by pulse-chase metabolic labeling as described for panel A. Anti-HA immunoprecipitates (Ip) were resolved by SDS-7.5% PAGE and revealed by fluorography. The position of HA-p105 is indicated with an arrow. (C) Cell lysates from the indicated HeLa clones with or without TNF-α stimulation (15 min) were Western blotted for endogenous IκBα. (D) Stably transfected HeLa cells were preincubated for 1 h with LLnL proteasome inhibitor and then stimulated for 15 min with TNF-α or control medium. Immunoprecipitates of wild-type and point-mutated HA-p105 were then sequentially Western blotted with the indicated antibodies.

To initially investigate the possibility of the signal-induced phosphorylation of p105 on serine 932, NIH 3T3 cells were transfected with plasmids encoding wild-type HA-p105 or HA-p105(S932A) plus FL-IKK2 or control empty vector (EV). Cells were incubated with MG132 inhibitor for the last 4 h of a 48-h culture to block proteasome-mediated proteolysis. HA-

p105 immunoprecipitated from cells coexpressing FL-IKK2 was clearly recognized by the anti-phospho-S<sup>932</sup>-p105 antibody but not from cells cotransfected with EV (Fig. 3A). However, no signal was obtained in cells cotransfected with HA-p105 (S932A) and FL-IKK2, although HA-p105(S932A) protein was present in immunoprecipitates and phosphorylated on

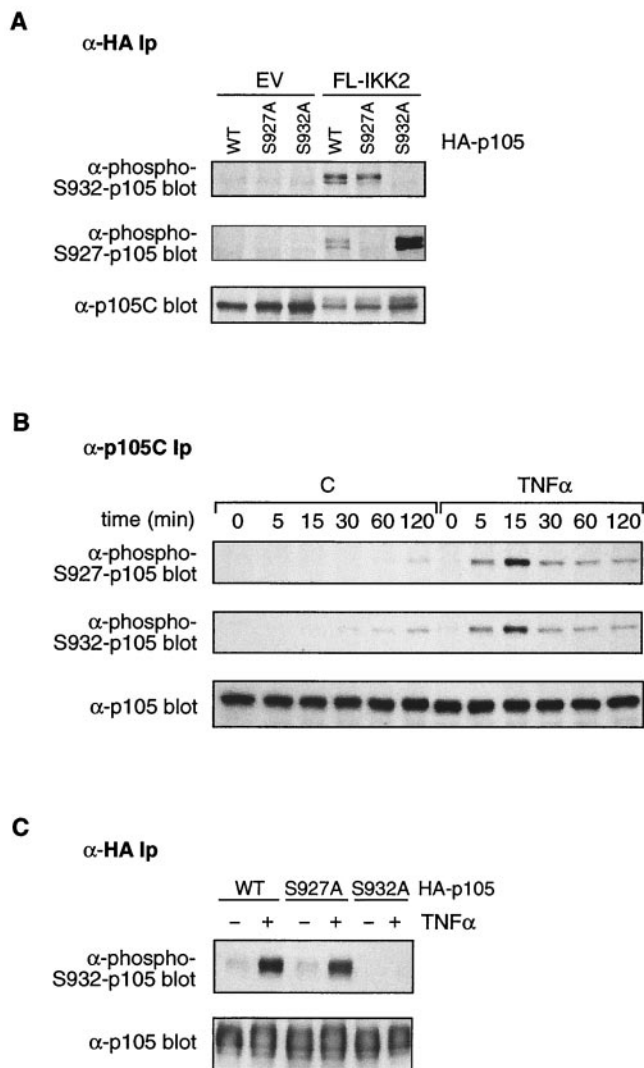


FIG. 3. TNF- $\alpha$  induces rapid phosphorylation of p105 on serine 932. (A) NIH 3T3 cells were transiently cotransfected with expression vectors encoding wild-type (WT) HA-p105 or the indicated mutants and IKK2 or EV and treated with MG132 proteasome inhibitor for the last 4 h of a 48-h culture. HA-p105 was immunoprecipitated, and isolated protein was sequentially Western blotted with anti-phospho-Ser<sup>932</sup> (upper gel), anti-phospho-Ser<sup>927</sup> (middle gel), and anti-p105C (lower gel) antibodies. Ip, immunoprecipitates. (B) HeLa cells were preincubated for 30 min with MG132 and then stimulated for the indicated times with TNF- $\alpha$  or control medium (C). Immunoprecipitates of endogenous p105 were then Western blotted sequentially with the indicated antibodies. (C) HeLa cells stably transfected with the indicated HA-p105 constructs were preincubated for 1 h with LLnL inhibitor and then stimulated for 15 min with TNF- $\alpha$  or control medium. Anti-HA immunoprecipitates were then sequentially Western blotted with the indicated antibodies.

serine 927. In addition, incubation of blots with anti-phospho-S<sup>932</sup>-p105 antibody plus S<sup>932</sup> phosphopeptide reduced the phospho-HA-p105 signal in FL-IKK2-cotransfected cells to background whereas the addition of unphosphorylated peptide had no effect (data not shown). Together, these data confirm the specificity of the anti-phospho-S<sup>932</sup>-p105 antibody and demonstrate that overexpression of FL-IKK2 induces the phosphorylation of HA-p105 on serine 932. Furthermore,

these data indicate that the reduced proteolysis of HA-p105 (S932A) in response to FL-IKK2 coexpression was not the consequence of decreased serine 927 phosphorylation (21). Conversely, the reduced IKK2-triggered proteolysis of HA-p105(S927A) did not result from the blockade of serine 932 phosphorylation (Fig. 3A).

To investigate whether proinflammatory cytokines induce the phosphorylation of serine 932 of p105, HeLa cells were preincubated with MG132 inhibitor for 30 min and then stimulated for the indicated times with TNF- $\alpha$ . Endogenous p105 was isolated from cell lysates by immunoprecipitation and then immunoblotted with anti-phospho-S<sup>932</sup>-p105 antibody. TNF- $\alpha$  stimulation induced rapid serine 932 phosphorylation that reached a maximum at 15 min and then declined (Fig. 3B, middle panel). The kinetics of p105 serine 927 phosphorylation after TNF- $\alpha$  stimulation were very similar (Fig. 3B, upper panel). Low levels of p105 phosphorylated on both serines 927 and 932 also accumulated in control unstimulated cells by 1 to 2 h, which were presumably due to basal IKK activity.

It was important to determine whether the inhibitory effect of the S927A mutation on the TNF- $\alpha$ -induced proteolysis of HA-p105 (21) was due to the indirect inhibition of serine 932 phosphorylation. To investigate this, HA-p105 was immunoprecipitated from the HeLa clone expressing HA-p105(S927A) and cultured for 1 h with LLnL proteasome inhibitor and for a further 15 min in the presence or absence of TNF- $\alpha$ . Western blotting with the anti-phospho-S<sup>932</sup>-p105 antibody indicated that HA-p105(S927A) was phosphorylated on serine 932 to a similar degree as wild-type HA-p105 phosphorylation after TNF- $\alpha$  stimulation (Fig. 3C). Thus, the S927A mutation does not inhibit the TNF- $\alpha$  induction of HA-p105 proteolysis by blocking serine 932 phosphorylation.

The data in this section confirm that serine 932 of p105 is phosphorylated under stimulatory conditions that promote p105 proteolysis and indicate that the inhibitory effect of the S932A mutation on TNF- $\alpha$ -induced p105 proteolysis is due to the removal of a regulatory phosphorylation site.

**Serine 932 of p105 is phosphorylated by the IKK complex.** Since the IKK complex is essential for signal-induced p105 proteolysis, it was important to determine whether cytokine-induced phosphorylation of p105 on serine 932 required the IKK complex. To investigate this, wild-type mouse EFs and IKK1/2 double knockout EFs (12), which lack a functional IKK complex, were pretreated for 30 min with MG132 inhibitor and then stimulated with TNF- $\alpha$  for 15 min. Western blotting of immunoprecipitated endogenous p105 indicated that TNF- $\alpha$  induced serine 932 phosphorylation in wild-type EF cells but not in the IKK1/2<sup>-/-</sup> cells (Fig. 4A). TNF- $\alpha$  also failed to induce serine 927 phosphorylation in the IKK1/2<sup>-/-</sup> EF cells, whereas serine 927 phosphorylation was induced in wild-type cells. Similar experiments with IKK1 or IKK2 single-knockout EFs demonstrated that IKK1 and IKK2 are redundant for the TNF- $\alpha$  induction of p105 phosphorylation on serines 927 and 932 (data not shown), consistent with the ability of TNF- $\alpha$  to induce p105 proteolysis in such cells (21). However, TNF- $\alpha$ -induced phosphorylation of these residues was consistently detected to be higher in wild-type cells than in the single knockouts, suggesting that IKK complexes containing both IKK1 and IKK2 are required for optimal p105 phosphorylation. TNF- $\alpha$  induction of p38 phosphorylation in the IKK1/2<sup>-/-</sup>

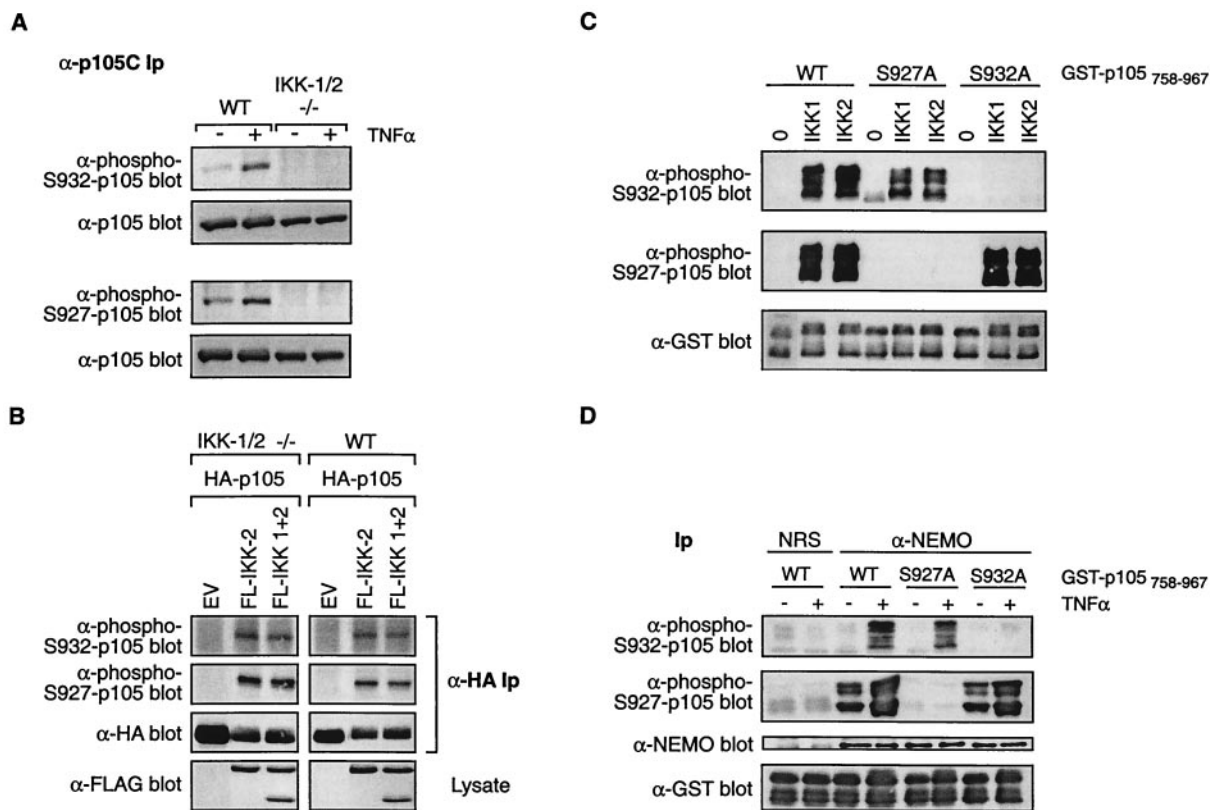


FIG. 4. The IKK complex directly phosphorylates serine 932 of p105. (A) Wild-type (WT) and *IKK1/2*<sup>-/-</sup> (-/-) EFs were pretreated with MG132 for 30 min and then incubated for a further 30 min with TNF-α or control medium. p105 was then immunoprecipitated from cell lysates and Western blotted with the indicated antibodies. Ip, immunoprecipitates. (B) *IKK1/2*<sup>-/-</sup> or wild-type EFs were transiently cotransfected with the indicated expression vectors. Cells were treated with MG132 for the last 4 h of a 48-h culture. HA-p105 was isolated from cell lysates by immunoprecipitation and immunoblotted with the antibodies shown. (C) In vitro kinase assays were carried out with baculovirus-produced purified His<sub>6</sub>-IKK1 or His<sub>6</sub>-IKK2 (100 ng), using as substrates 5 μg of wild-type or point-mutated GST-p105<sub>758-967</sub> protein, as indicated. Phosphorylation was assessed by sequentially immunoblotting with anti-phospho-Ser<sup>932</sup> (upper gel) or anti-phospho-Ser<sup>927</sup> (middle gel) antibodies. Equal loading of fusion proteins was confirmed by reprobing blots with anti-GST MAb (lower gel). (D) HeLa cells were stimulated for 15 min with TNF-α or left unstimulated. The endogenous IKK complex was isolated from cell lysates by immunoprecipitation with an anti-NEMO antibody. Control immunoprecipitations were carried out with nonimmune rabbit serum (NRS). Phosphorylation was assessed as described for panel C. Blots were probed with anti-NEMO antibody to confirm that equal amounts of IKK complex were immunoprecipitated.

EFs was similar to that in the wild type, confirming that the TNF-α signaling was intact (data not shown). Furthermore, transient overexpression of FL-IKK2 or FL-IKK1 and FL-IKK2 in the *IKK1/2*<sup>-/-</sup> EFs strongly induced phosphorylation of p105 on both serines 927 and 932 (Fig. 4B), supporting the hypothesis that the defect in TNF-α-induced p105 phosphorylation in these cells is indeed due to the lack of a functional IKK complex.

These data raise the possibility that the IKK complex might directly phosphorylate p105 serine 932. To investigate this, GST fusion proteins were purified from *Escherichia coli* encoding the C terminus of p105 (residues 758 to 967; GST-p105<sub>758-967</sub>) and corresponding mutants in which the residues equivalent to p105 serine 927 or 932 were mutated to alanine [GST-p105<sub>758-967</sub>(S927A) and GST-p105<sub>758-967</sub>(S932A), respectively]. These fusion proteins were phosphorylated in vitro with baculovirus-expressed His<sub>6</sub>-IKK1 or His<sub>6</sub>-IKK2. Western blotting with anti-phospho-S<sup>932</sup>-p105 antibody confirmed that both kinases phosphorylated GST-p105<sub>758-967</sub> and GST-p105<sub>758-967</sub>(S927A) on serine 932, whereas no signal was de-

tected with GST-p105<sub>758-967</sub>(S932A), as expected (Fig. 4C). Similarly, both His<sub>6</sub>-IKK1 and His<sub>6</sub>-IKK2 also phosphorylated serine 927 of the wild type and the S932A mutants of GST-p105<sub>758-967</sub> but not of the S927A mutant, as reported previously (21). Endogenous IKK complex, purified by immunoprecipitation from TNF-α-stimulated HeLa cells, also phosphorylated both serine 927 and serine 932 of p105 (Fig. 4D). Thus, p105 serine 932 is a direct target of the IKK complex in vitro. The combined biochemical and genetic data in this section therefore demonstrate that TNF-α induces the phosphorylation of both serine 927 and serine 932 of p105 via the IKK complex.

**Signal-induced phosphorylation of serine 927 and serine 932 regulates p105 ubiquitination.** IKK2-induced phosphorylation of p105 triggers its ubiquitination, targeting it for proteolysis by the proteasome (8, 19). The role of the phosphorylation of serines 927 and 932 on TNF-α-induced p105 ubiquitination was therefore investigated. HeLa cells stably transfected with HA-p105, HA-p105(S927A), or HA-p105(S932A) were pretreated with the LLnL proteasome inhibitor

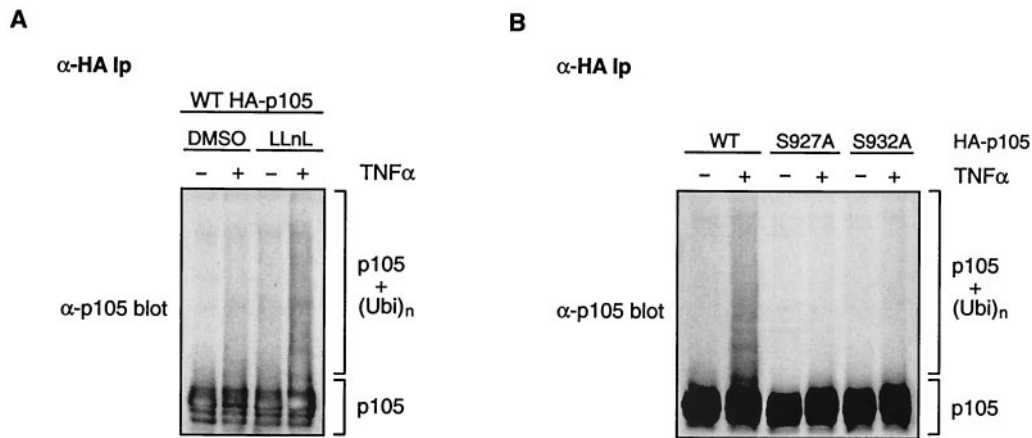


FIG. 5. Serines 927 and 932 are required for TNF- $\alpha$  to trigger p105 ubiquitination. (A) HeLa cells stably transfected with HA-p105 were pretreated for 1 h with LLnL proteasome inhibitor or vehicle control (dimethyl sulfoxide [DMSO]). Cells were then stimulated for 15 min with TNF- $\alpha$  or control medium. HA-p105 was isolated from cell lysates by immunoprecipitation, resolved by SDS-6% PAGE, and then Western blotted with anti-p105C antibody. Ip, immunoprecipitates; WT, wild type. (B) The indicated HeLa clones were pretreated with LLnL inhibitor and stimulated with TNF- $\alpha$  as described for that in panel A. Anti-HA immunoprecipitates were Western blotted with anti-p105C antibody.

for 1 h. The cells were then incubated for a further 15 min with TNF- $\alpha$  or left unstimulated. HA-p105, immunoprecipitated from cell lysates with anti-HA MAb, was Western blotted with anti-p105C antibody. TNF- $\alpha$  stimulation promoted the appearance of a high-molecular-weight ladder of more slowly migrating forms of HA-p105, characteristic of ubiquitinated p105 (8, 19), which were not visible if LLnL inhibitor was omitted (Fig. 5A). Direct immunoblotting of p105 immunoprecipitates with antiubiquitin antibody confirmed that TNF- $\alpha$ -induced laddering was due to HA-p105 polyubiquitination (data not shown). However, TNF- $\alpha$  stimulation failed to induce high-molecular-weight forms of either HA-p105(S927A) or HA-p105(S932A) (Fig. 5B). These data indicate that both the S927A and S932A mutations block the TNF- $\alpha$ -induced ubiquitination of p105, suggesting that the phosphorylation of both serines 927 and 932 is required for TNF- $\alpha$  to trigger p105 ubiquitination. This is consistent with the observation that serines 927 and 932 are both essential for TNF- $\alpha$ -induced p105 proteolysis (Fig. 2A).

**$\beta$ TrCP is essential for the TNF- $\alpha$  induction of p105 and I $\kappa$ B $\alpha$  proteolysis.** Biochemical experiments have demonstrated that the phosphorylation of p105 by IKK creates a binding site for  $\beta$ TrCP1 and/or  $\beta$ TrCP2, F-box WD40 repeat proteins that are the receptor subunits of an SCF-type E3 ubiquitin ligase (19). In vitro experiments indicate that this association promotes the polyubiquitination of p105 and subsequent proteolysis by the proteasome (8, 19). Consistent with this model, the transient expression of an F-box deletion mutant of  $\beta$ TrCP1, which can bind to IKK-phosphorylated p105 but cannot assemble with other SCF subunits, blocks p105 proteolysis induced by IKK2 overexpression (19).

To determine whether TNF- $\alpha$  stimulation also induces binding of  $\beta$ TrCP (used as a collective term referring to both  $\beta$ TrCP1 and  $\beta$ TrCP2) to p105, HeLa cells stably expressing HA-p105 were transiently transfected with expression vectors encoding  $\beta$ TrCP1 and  $\beta$ TrCP2. HA-p105 was isolated by immunoprecipitation from lysates of cells stimulated for 15 min with TNF- $\alpha$  or left unstimulated. TNF- $\alpha$  stimulation induced a

clear association between HA-p105 and both  $\beta$ TrCP1 and  $\beta$ TrCP2 (Fig. 6A).

Functional experiments on the role of  $\beta$ TrCP on p105 ubiquitination have relied on purified components for in vitro experiments or protein overexpression in cells (8, 19). It was therefore important to confirm genetically that  $\beta$ TrCP was indeed required for TNF- $\alpha$ -induced p105 proteolysis. siRNA-mediated gene suppression (6), based on a 21-bp double-stranded RNA complementary to both  $\beta$ TrCP1 and  $\beta$ TrCP2, was used to codeplete  $\beta$ TrCP1 and  $\beta$ TrCP2 from HeLa cells stably transfected with HA-p105. Western blotting confirmed that siRNA depleted  $\beta$ TrCP expression to undetectable levels (Fig. 6B, upper panel). Pulse-chase metabolic labeling revealed that  $\beta$ TrCP depletion blocked TNF- $\alpha$ -induced HA-p105 proteolysis (Fig. 6C). TNF- $\alpha$ -induced p105 ubiquitination, as revealed by the presence of high-molecular-weight p105 laddering, was also substantially reduced by  $\beta$ TrCP depletion (Fig. 6D). In addition, TNF- $\alpha$ -induced I $\kappa$ B $\alpha$  proteolysis was blocked in HeLa cells depleted of  $\beta$ TrCP (Fig. 6B, lower panel) whereas TNF- $\alpha$ -induced p38 phosphorylation was unaffected (data not shown). Thus,  $\beta$ TrCP is required for TNF- $\alpha$  to promote the ubiquitination and proteolysis of p105, supporting earlier models for  $\beta$ TrCP in regulating p105 function (8, 19). Furthermore, these experiments confirm the essential role of  $\beta$ TrCP in TNF- $\alpha$ -induced I $\kappa$ B $\alpha$  degradation (4).

The effect of  $\beta$ TrCP depletion on TNF- $\alpha$ -induced p105 phosphorylation was also investigated. TNF- $\alpha$ -induced phosphorylation of p105 on serines 927 and 932 was substantially increased in  $\beta$ TrCP-depleted cells compared to that in the control (Fig. 6E). This suggests that p105 phosphorylated on serines 927 and 932 is specifically targeted for ubiquitination by  $\beta$ TrCP, consistent with the essential role of these residues in TNF- $\alpha$ -induced p105 ubiquitination and proteolysis (Fig. 2A and 5B).

**Efficient binding of  $\beta$ TrCP to p105 requires phosphorylation of both serines 927 and 932.** The siRNA experiments raise the possibility that the recruitment of  $\beta$ TrCP to p105 requires phosphorylation on both serines 927 and 932. A competition

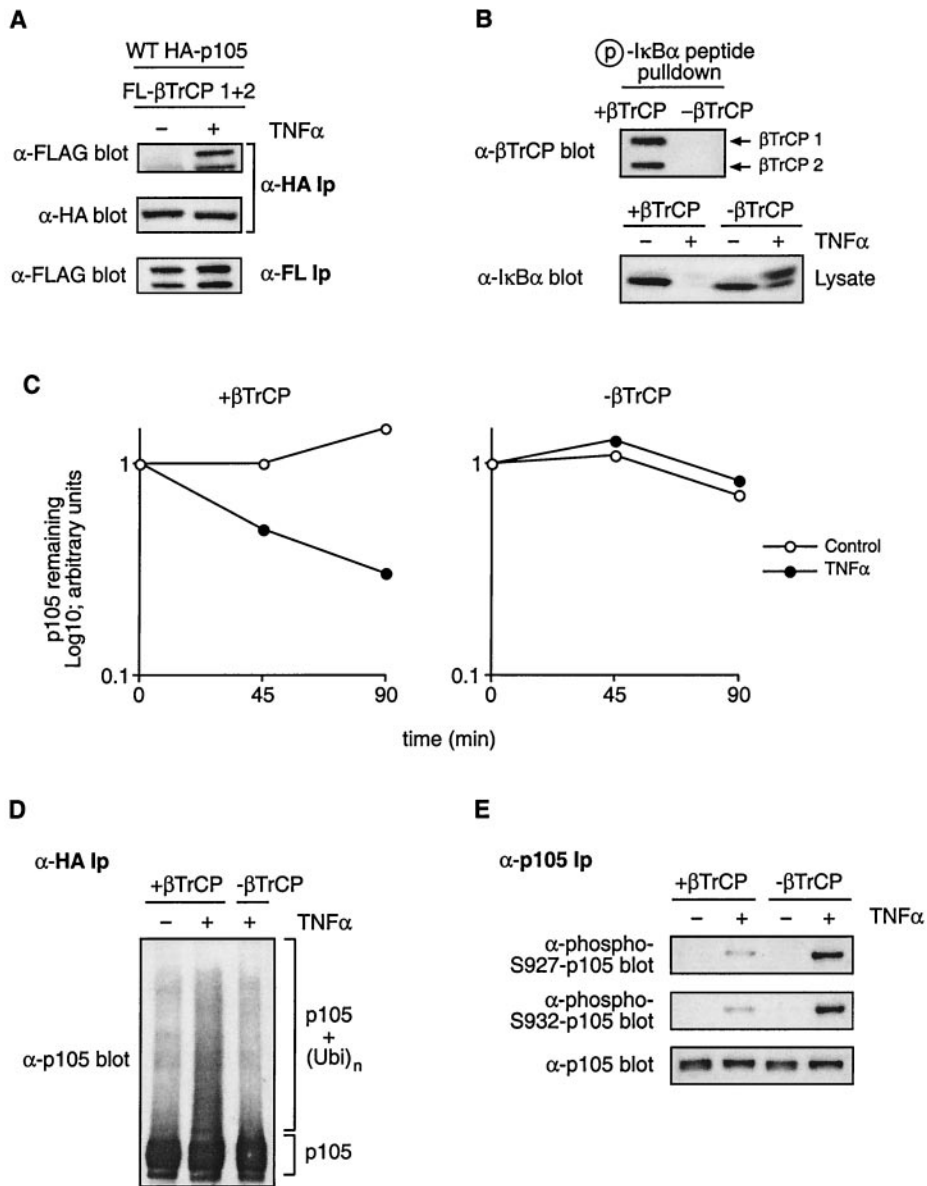


FIG. 6.  $\beta$ TrCP is required for TNF- $\alpha$ -induced p105 ubiquitination and proteolysis. (A) HeLa cells stably transfected with HA-p105 were transiently transfected with vectors encoding FL- $\beta$ TrCP1 and FL- $\beta$ TrCP2. After 48-h culture, cells were stimulated for 15 min with TNF- $\alpha$  or left unstimulated. Anti-HA immunoprecipitates (Ip) were Western blotted with the indicated antibodies. FL- $\beta$ TrCP expression in cell lysates was confirmed by anti-FL MAb immunoprecipitation and Western blotting. WT, wild type. (B) HeLa cells were pretreated with  $\beta$ TrCP siRNAs complementary to both  $\beta$ TrCP1 and  $\beta$ TrCP2 ( $-\beta$ TrCP) or with control buffer ( $+\beta$ TrCP). After 72 h, cells were stimulated with TNF- $\alpha$  (15 min) or left unstimulated and then lysed. In the upper gel,  $\beta$ TrCP expression of unstimulated cells was determined by Western blotting of phospho-I $\kappa$ B $\alpha$  peptide pull-downs. In the lower gel, cell lysates were Western blotted for I $\kappa$ B $\alpha$ . (C) HeLa cells stably expressing HA-p105 were pretreated as described for panel B. Turnover of HA-p105 in TNF- $\alpha$ -stimulated and control unstimulated cells was determined by pulse-chase metabolic labeling as done for that shown in Fig. 2. Similar results were obtained on two other occasions. (D)  $\beta$ TrCP expression in HA-p105 HeLa cells was suppressed by siRNA pretreatment as described for panel B. HA-p105 ubiquitination induced by 15-min TNF- $\alpha$  stimulation was determined as described in the legend to Fig. 5. (E)  $\beta$ TrCP expression was decreased by siRNA treatment as described for panel B. Phosphorylation of endogenous p105 after TNF- $\alpha$  treatment (15 min) was determined by immunoprecipitation and Western blotting with the indicated antibodies.

assay using synthetic phosphopeptides (Fig. 1C) corresponding to residues 922 to 935 of p105 was used to directly determine the role of p105 serine 927 and 932 phosphorylation in  $\beta$ TrCP binding.

For this, GST-p105<sub>758-967</sub> fusion protein, phosphorylated in vitro by recombinant His<sub>6</sub>-IKK2 or left untreated, was coupled to glutathione-Sepharose beads and used as an affinity matrix

to isolate [<sup>35</sup>S]methionine-labeled  $\beta$ TrCP1 and  $\beta$ TrCP2 which had been translated in vitro. As expected, the amount of  $\beta$ TrCP isolated by GST-p105<sub>758-967</sub> was significantly increased following His<sub>6</sub>-IKK2 phosphorylation compared with that for the control unphosphorylated protein (Fig. 7A). The addition of phosphoserine 932 p105<sub>922-935</sub> peptide had no effect on  $\beta$ TrCP binding compared to no peptide (data not shown) or



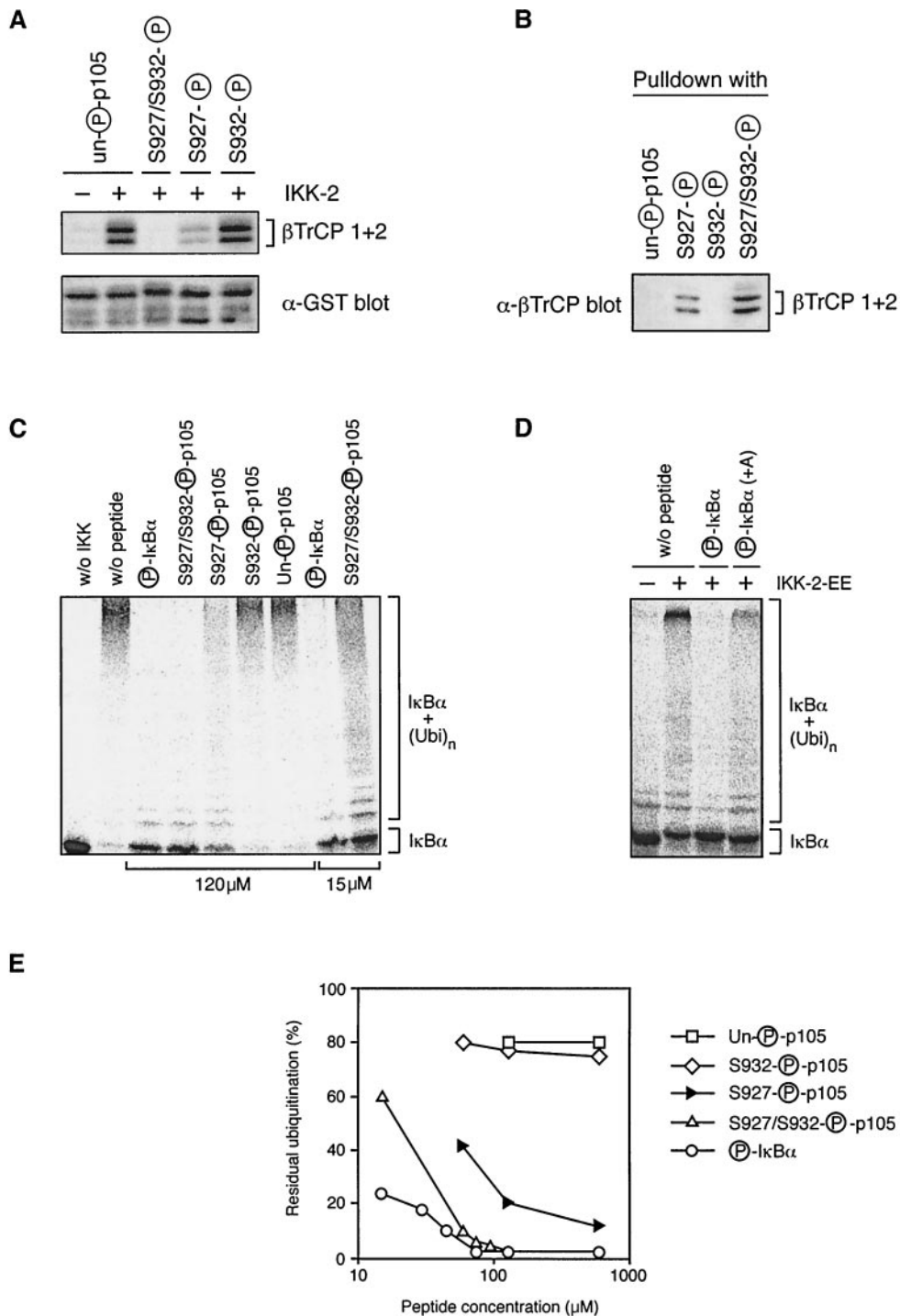


FIG. 7. Efficient binding of  $\beta$ TrCP to p105 requires phosphorylation of p105 serines 927 and 932. (A) GST-p105<sub>758-967</sub> fusion protein, phosphorylated by His<sub>6</sub>-IKK2 or left unphosphorylated, was coupled to glutathione-Sepharose beads and used to affinity purify  $\beta$ TrCP translated and labeled with [<sup>35</sup>S]methionine in vitro. The indicated peptides were added to a final concentration of 100  $\mu$ g/ml during the pulldown. Isolated  $\beta$ TrCP was detected by autoradiography of SDS-8% PAGE gels. (B) The indicated peptides were coupled to Affi-Gel 10 beads and used as affinity matrices to isolate from HeLa cell lysates endogenous  $\beta$ TrCP, which was detected by Western blotting. (C and D) I $\kappa$ B $\alpha$ , translated and labeled in vitro with [<sup>35</sup>S]methionine, was first phosphorylated with recombinant IKK2-EE. Labeled protein was then ubiquitinated in vitro (26) in the presence of the indicated peptides or with no added peptide (w/o peptide). Ubiquitination of unphosphorylated I $\kappa$ B $\alpha$  (w/o IKK) is shown as a negative control. I $\kappa$ B $\alpha$  ubiquitination was revealed by autoradiography of SDS-8% PAGE gels. (E) The extent of I $\kappa$ B $\alpha$  ubiquitination in vitro (carried out as described for panel C) was quantified on a phosphorimager over a range of competing peptide concentrations.

to the addition of unphosphorylated p105<sub>922-935</sub> peptide. Some inhibition of binding was detected upon addition of p105<sub>922-935</sub> peptide phosphorylated on serine 927. However, addition of p105<sub>922-935</sub> peptide phosphorylated on both serines 927 and 932 completely blocked binding to βTrCP.

These data suggest that p105 requires phosphorylation on both serines 927 and 932 to most efficiently bind βTrCP but that weak binding can be achieved when only serine 927 is phosphorylated. To investigate this possibility directly, each of the phosphopeptides was individually coupled to Affi-Gel 10 beads and then used as affinity matrices to purify endogenous βTrCP from HeLa cell lysates. Western blotting revealed no βTrCP binding with unphosphorylated p105<sub>922-935</sub> peptide or phosphoserine 932 p105<sub>922-935</sub> peptide (Fig. 7B), consistent with the peptide competition experiment (Fig. 7A). However, βTrCP binding was detected to p105<sub>922-935</sub> peptide phosphorylated on only serine 927, but to a lesser extent than that to the doubly phosphorylated p105<sub>922-935</sub> peptide (Fig. 7B). These data confirm that βTrCP binds more effectively to p105 phosphorylated on both serines 927 and 932.

**βTrCP has a higher affinity for phospho-IκBα than phospho-p105.** Previous experiments have indicated that p105 and IκBα are phosphorylated with very similar kinetics by IKK after TNF-α stimulation of HeLa cells (21). However, TNF-α stimulation only results in the slow and partial degradation of p105, in contrast to IκBα, whose degradation is very rapid and complete (Fig. 2C) (21). A possible explanation for this difference is that the two proteins recruit SCF<sup>βTrCP</sup> with different efficiencies after IKK-mediated phosphorylation and, as a result, are ubiquitinated at different rates.

A previously developed *in vitro* IκBα ubiquitination assay (26) was used to compare the relative affinity of βTrCP for the IKK target sites on p105 and IκBα. *In vitro*-translated and [<sup>35</sup>S]methionine-labeled IκBα was allowed to associate with immunopurified NF-κB (from HeLa cells), phosphorylated with recombinant IKK2-EE, and subjected to βTrCP-mediated ubiquitination in a cell-free system which does not support IκBα phosphorylation or proteasome-mediated degradation. Under control conditions, phosphorylated IκBα, but not unphosphorylated IκBα, was efficiently converted to a polyubiquitinated species (Fig. 7C, compare lane without IKK to lane without peptide). The addition of IκBα<sub>28-37</sub> peptide phosphorylated on serines 32 and 36 (Fig. 1C) completely inhibited IκBα ubiquitination with a 50% inhibitory concentration (IC<sub>50</sub>) of ≤5 μM (Fig. 7C and E). The addition of p105<sub>922-935</sub> peptide phosphorylated on serines 927 and 932 also inhibited IκBα ubiquitination but was much less efficient (IC<sub>50</sub> ≈ 25 μM) than the phospho-IκBα<sub>28-37</sub> peptide. The phosphoserine 927 p105<sub>922-935</sub> peptide was even less effective (IC<sub>50</sub> ≈ 60 μM) in inhibiting IκBα ubiquitination, and phosphoserine 932 p105<sub>922-935</sub> peptide had no detectable effect (Fig. 7C and E). These data confirm that βTrCP preferentially binds to p105 phosphorylated on both serines 927 and 932 and also indicate that βTrCP has a higher affinity for phospho-IκBα than for phospho-p105.

The Asp-Ser<sup>927</sup>(P)-Gly-Val-Glu-Thr-Ser<sup>932</sup>(P) motif in the PEST region of p105 is closely related to the N-terminal IKK target sequence of IκBα, Asp-Ser<sup>32</sup>(P)-Gly-Leu-Asp-Ser<sup>36</sup>(P), except for the introduction of an extra residue between the two phosphorylated serines in p105 (Fig. 1A). To determine

whether this spacing might explain the differential affinity of βTrCP for the phospho-IκBα<sub>28-37</sub> and phospho-p105<sub>922-935</sub> peptides, an IκBα<sub>28-37</sub> phosphopeptide was synthesized in which the spacer between phosphoserines 32 and 36 was increased by the introduction of an alanine residue (Fig. 1C). This peptide was less effective in blocking IκBα ubiquitination than the wild-type phospho-IκBα<sub>28-37</sub> peptide (Fig. 7D) (IC<sub>50</sub> ≈ 50 μM estimated from titration experiments). This suggests that the high affinity binding of βTrCP to phosphorylated targets requires a 3-amino-acid spacing between phosphorylated serines and that target proteins with a 4-amino-acid spacing, such as p105, bind with lower affinity.

## DISCUSSION

The experiments in this study demonstrate that phosphorylation of serines 927 and 932 within the PEST region of NF-κB1 p105 is essential for its degradation triggered by TNF-α stimulation (Fig. 2A). This reflects a requirement for the phosphorylation of both serines to promote efficient recruitment of the E3 ubiquitin ligase SCF<sup>βTrCP</sup> to phospho-p105 and subsequent p105 ubiquitination (Fig. 6). Furthermore, *in vitro* experiments demonstrate that serine 927 and serine 932 are directly phosphorylated by the IKK complex, whose expression is necessary for the TNF-α-induced phosphorylation of both serines in EF cells (Fig. 4). IL-1 stimulation also triggers both serine 927 and 932 phosphorylation of p105, and IL-1-induced p105 proteolysis is blocked by the mutation of either serine 927 or serine 932 to alanine (21) (data not shown). Thus, although the signaling pathways triggered by TNF-α and IL-1 are distinct (18, 25), both cytokines appear to trigger p105 proteolysis via a common phosphorylation-based mechanism. It is likely that p105 proteolysis induced by other agonists, such as lipopolysaccharide and phorbol ester (5, 14), will also involve the phosphorylation of serines 927 and 932 by the IKK complex. Consistent with this hypothesis, p105 proteolysis triggered by overexpressed TPL2/Cot (3) also requires the phosphorylation of both serines 927 and 932 (data not shown).

Heissmeyer et al. have previously suggested a regulatory role for serine 923 (Asp-Ser<sup>923</sup>-Val-Cys-Asp-Ser<sup>927</sup>) in IKK2-induced HA-p105 proteolysis in 293 cells transiently cotransfected with βTrCP (8). However, p105 degradation triggered by phosphorylation by the endogenous IKK complex after TNF-α stimulation does not require serine 923 (Fig. 2A) and an earlier study by our laboratory also failed to reveal a role for this residue in IKK2-induced p105 proteolysis in NIH 3T3 cells (21). Serine 923, therefore, is unlikely to be an important physiological target of the IKK complex, and it has not yet been demonstrated that serine 923 is actually phosphorylated after cytokine stimulation. To investigate this directly, an antibody was raised to a synthetic peptide corresponding to residues 921 through 933 of human p105 in which serine 923 is phosphorylated. Although this antibody clearly recognized specifically the immunizing phosphopeptide on dot blots, it did not bind to p105 phosphorylated either *in vivo* after TNF-α stimulation or *in vitro* by recombinant IKK2 (data not shown). These latter results contrast with those of Heissmeyer et al. (8), who reported that purified IKK2 phosphorylated the recombinant His<sub>6</sub>-p105<sup>917-968</sup> fusion protein on serine 923 *in vitro*. The Ley laboratory has recently demonstrated that the efficient

phosphorylation of p105 on both serines 927 and 932 in vitro requires the p105 death domain (p105 residues 802 to 893), which acts as a docking site for IKK2 (2) (data not shown). It is possible that serine 923 of the recombinant His<sub>6</sub>-p105<sup>917-968</sup> fusion protein is artefactually phosphorylated by IKK2 due to the absence of p105 death domain-IKK2 interaction, which may be important both to ensure the efficiency of phosphorylation and for correct target amino acid specificity (2).

The Asp-Ser<sup>927</sup>-Gly-Val-Glu-Thr-Ser<sup>932</sup> motif in the PEST domain of p105 is distinct from the N-terminal IKK target sequence of IκBα (Asp-Ser<sup>32</sup>-Gly-Leu-Asp-Ser<sup>36</sup>) due to the introduction of an extra residue between the two phosphorylated serines in p105 (Fig. 1A). In vitro binding experiments suggest that one functional consequence of this extra amino acid in p105 is to significantly decrease the affinity of βTrCP for the IKK phosphorylated sequence (Fig. 7). Consistent with this possibility, introduction of an extra amino acid between the IKK-phosphorylated serines in IκBα reduces the ability of IκBα phosphopeptides to block βTrCP-dependent IκBα ubiquitination (Fig. 7D). Interestingly, the IKK target sequence in p105 is completely conserved between human, mouse, rat, and chicken p105 (21), suggesting that the lower affinity of phospho-p105 for βTrCP confers some evolutionary advantage. Presumably one consequence of this decreased binding is to reduce the efficiency of βTrCP-mediated ubiquitination of phospho-p105 compared with that of phospho-IκBα. This may explain in part the slow and partial proteolysis of p105 in TNF-α-stimulated cells compared with that of IκBα, which is rapidly and completely degraded (21). However, although IKK phosphorylates p105 and IκBα with similar kinetics after TNF-α stimulation (21), it cannot be excluded that the IKK complex phosphorylates IκBα at a higher stoichiometry than p105, and this may also contribute to the different efficiency of their proteolysis in TNF-α-stimulated cells.

Both phosphopeptide competition and pull-down experiments (Fig. 7) indicate that βTrCP can bind weakly when p105 serine 927 alone is phosphorylated, whereas no binding is detected to a phosphoserine 932 p105 peptide. However, phosphorylation of both serines 927 and 932 generates a higher affinity binding site for βTrCP. Interestingly binding of βTrCP1 to phospho-IκBα peptides shows a similar phosphorylation requirement. Thus, an IκBα peptide phosphorylated on both serines 32 and 36 binds to recombinant βTrCP1 with high affinity ( $K_d = 70$  nM) whereas a peptide phosphorylated on only serine 32 binds much more weakly ( $K_d = 16$  μM) (R. Hay, unpublished data). In contrast, binding to a phosphoserine 36 IκBα peptide is unmeasurable. Together these data suggest that βTrCP has a bipartite phosphopeptide binding site in which the N-terminal phosphoserine of either p105 or IκBα interacts more strongly than the C-terminal phosphoserine.

Heissmeyer et al. have reported that HA-p105 degradation triggered by coexpression with FL-IKK2 and βTrCP1 in 293 cells is unaffected by the introduction of a serine 932-to-alanine mutation but blocked by a serine 927-to-alanine mutation (8). Thus, HA-p105 proteolysis promoted by overexpressed FL-IKK2 and βTrCP1 can be triggered by serine 927 phosphorylation alone. In contrast, a serine 932-to-alanine mutation delays HA-p105 proteolysis promoted by FL-IKK2 expression in NIH 3T3 fibroblasts (21) and completely blocks p105 proteolysis triggered by TNF-α stimulation (Fig. 2A). Thus, in

these latter experimental models, HA-p105 must be phosphorylated on both serines 927 and 932 for optimal signal-induced proteolysis. It is likely that βTrCP1 overexpression masks the need for serine 932 phosphorylation by artefactually promoting sufficient βTrCP1 binding to HA-p105 that is phosphorylated only on serine 927 (Fig. 7B). However, when ubiquitination is mediated by endogenous SCF<sup>βTrCP</sup>, phosphorylation of both serines is required for optimal βTrCP binding. The present study therefore highlights the importance of determining the function of phosphorylation sites on a protein where the participating regulatory proteins (i.e., IKK and SCF<sup>βTrCP</sup>) are expressed at physiological levels, rather than relying solely on transient overexpression experiments.

In conclusion, this study demonstrates that the TNF-α-induced proteolysis of NF-κB1 p105 involves the IKK complex-mediated phosphorylation of serines 927 and 932 in the PEST region, within a motif similar to that in the IKK target sequence of IκBα. Phosphorylation of both residues is shown to be required for the efficient recruitment of βTrCP, a subunit of the SCF-type E3 ligase which promotes the ubiquitination of phospho-p105 and triggers its subsequent proteolysis.

#### ACKNOWLEDGMENTS

V.L. and J.J. contributed equally to this work.

We thank Nancy Bump, Alain Israel, Frank Mercurio, Keiji Tanaka, and Inder Verma for the reagents used in this study. The help of Lee Johnston, Steve Smerdon, and Steve Gamblin (National Institute for Medical Research) for suggestions on the manuscript, Sandra Watton (National Institute for Medical Research) for advice on affinity purification of antiphosphopeptide antibodies, and Lesley Thompson (University of St. Andrews) and Ada Hatzubai (Hebrew University) for helping with in vitro ubiquitination experiments is gratefully acknowledged. We are also indebted to the PhotoGraphics department (National Institute for Medical Research) for making the figures.

This work was supported by the U.K. Medical Research Council, the Arthritis Research Campaign (project grant L0536 to V.L.), and the AINP consortium, EC—5th framework.

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