The Death Domain of NF-κB1 p105 Is Essential for Signal-induced p105 Proteolysis*

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Stimulation of cells with tumor necrosis factor α (TNFα) triggers NF-κB1 p105 proteolysis, releasing associated Rel subunits to translocate into the nucleus and modulate target gene expression. Phosphorylation of serine 927 within the p105 PEST region by the IκB kinase (IKK) complex is required to promote p105 proteolysis in response to TNFα stimulation. In this study, the role of the p105 death domain (DD) in signal-induced p105 proteolysis is investigated. Endogenous p105 is shown to interact with the IKK complex in HeLa cells, and transient transfection experiments in 293 cells indicate that each of the catalytic components of the IKK complex, IKK1 and IKK2, can bind to p105. Interaction of p105 with both IKK1 and IKK2 is substantially reduced by deletion of the p105 DD or introduction of a specific point mutation (L841A) into the p105 DD homologous to the lpr mutation in Fas. Phosphorylation of immunoprecipitated p105 on serine 927 by purified recombinant IKK1 or IKK2 protein in vitro is dramatically reduced in both DD mutants relative to wild type. Furthermore, both of the DD mutations significantly impair the ability of low concentrations of IKK2 to induce p105 serine 927 phosphorylation and proteolysis in transiently transfected 3T3 cells. However, high levels of transiently expressed IKK2 bypass the requirement for the p105 DD to induce p105 serine 927 phosphorylation. Finally, p105 serine 927 phosphorylation by the endogenous IKK complex after TNFα stimulation and subsequent p105 proteolysis is blocked in both p105 DD mutants when stably expressed in HeLa cells. Thus, the p105 DD acts as a docking site for IKK, increasing its local concentration in the vicinity of the p105 PEST region and facilitating efficient serine 927 phosphorylation.

The nuclear factor (NF)-κB family of transcription factors includes Rel-A, Rel-B, c-Rel, NF-κB1 p50, and NF-κB2 p52 (1). These proteins bind DNA as homo- and heterodimers and play an important role in the inducible expression of genes involved in immune and inflammatory responses, apoptosis, development, and malignant transformation. NF-κB dimers are regulated primarily by binding to inhibitory proteins, the IκBs, which retain them in the cytoplasm of unstimulated cells (2). In response to stimulation with agonists, such as the proinflammatory cytokine tumor necrosis factor α (TNFα), IκBs are phosphorylated by the IκB kinase (IKK) complex, which contains two catalytic subunits, IKK1 (IKKe) and IKK2 (IKKβ), and a structural subunit NEMO (IKKγ) (2, 3). This triggers IκB ubiquitination and subsequent degradation by the proteasome, releasing associated NF-κB dimers to translocate into the nucleus and modulate target gene expression. In addition, the transcriptional activity of nuclear NF-κB dimers is modified by association with other DNA binding proteins, which is regulated by Rel subunit phosphorylation (1).

Two of the active subunits of NF-κB, NF-κB1 p50 and NF-κB2 p52, are produced as larger inactive precursors of 105 kDa (p105) and 100 kDa (p100), respectively (4, 5). p50 and p52 are derived from the N-terminal domain of their precursor molecules via post-translational processing of the C-terminal, ankyrin repeat-containing domain by the 26 S proteasome (6, 7). It has also been suggested that p105 and p100 are processed co-translationally to produce p50 and p52, respectively (8, 9). However, the importance of this processing mechanism is currently unclear. A Gly-rich region is required for processing of p105 to p50, which appears to function by blocking entry of the p50 fragment of p105 into the proteasome and preventing complete p105 degradation (10, 11). The Gly-rich region in p100 has a similar function in p52 production from p100 (8).

Processing of p105 to p50 occurs constitutively but is inefficient, and the majority of p105 is simply slowly degraded (2). Therefore, cellular levels of p105 are determined by two proteolytic pathways: limited (processing to p50) and complete (degradation). Unprocessed p105 functions as an IκB, which is thereby retained in the cytoplasm (12, 13). The C-terminal PEST region of p105 contains a conserved motif (Asp-Ser927-Gly-Val-Thr-Ser) that is related to the IKK target sequence in IκBα (3, 14). Following stimulation with TNFα, and other NF-κB agonists, p105 is phosphorylated on serine 927 by the IKK complex (3). This leads to the recruitment of the SCF⁶⁷−⁷⁸ ubiquitin ligase complex to p105, which is then ubiquitinated and proteolyzed by the proteasome (14, 15). The increase in p50 is relatively modest compared with the clear decrease in p105 levels, suggesting that IKK-mediated phosphorylation predominantly promotes degradation rather than processing (16, 17). Degradation of p105 releases p50 and other associated Rel subunits to translocate into the nucleus and modulate target gene transcription. Analysis of knockout mice that lack the C-terminal (IκB-like) half but still express p50 has indicated an essential role for p105 in the proper regulation of p50 homodimers, which is required for correct control of inflammatory responses (18).
half, which is N-terminal to the IKK phosphorylation site (19). One of the established functions of the DD motif is to mediate homo- and heterotypic protein interactions between receptors and adapters involved in signaling pathways regulating apoptosis, NF-κB and AP-1 (20–22). In this study, both IKK1 and IKK2 are shown to associate with p105, and these interactions are demonstrated to require a functional p105 DD. These findings are consistent with a recent study by Heissmeyer et al. (14) published while this work was in progress. Furthermore, functional experiments in the present study reveal for the first time that recruitment of IKK to p105 by the p105 DD is essential to facilitate signal-induced p105 serine 927 phosphorylation and subsequent proteolysis.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs and Antibodies—HA epitope-tagged p105 (HA-p105) cDNAs were cloned into the pcDNA3 expression vector (Invitrogen) for transient expression experiments and into the pMX-1 expression vector (Ingenus) for generation of stably transfected HeLa cell lines (3). Deletion and point mutants of HA-p105 and N-terminally Myc epitope-tagged p105 (Myc-p105) were generated using PCR and verified by DNA sequencing. Myc-p105 was cloned into the pcDNA3 vector. Expression vectors encoding HA epitope-tagged NEMO (HA-NEMO) and wild-type and kinase-inactive FLAG epitope-tagged IKK1 and IKK2 (FL-IKK1/2) have been described previously (23–25).

12CA5 mAb was used for immunoprecipitation of HA-p105, whereas a high affinity anti-HA mAb 3F10 (Roche Molecular Biochemicals) was used for Western blot detection of HA-tagged proteins. Myc-p105 was immunoprecipitated using 9E10 mAb and detected in Western blots using a commercial anti-Myc antibody (sc-372; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). M2 mAb (Sigma) was used for Western blotting of FLAG-tagged proteins. Anti-p105C and anti-phospho-Ser927, p105 antibodies have been described in a previous paper (3). The anti-IKK1/2 antibody was raised against a peptide sequence present in both IKK1 and IKK2 (3) and therefore directly recognizes both proteins.

**Lines and Transfections—**HeLa cells (Ohio subline from ECACC) and 293 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) for 1 h. Cells were pulse-labeled with 2.65 MBq of [35S]methionine/[35S]cysteine (Pre-Mix; Amersham Pharmacia Biotech) for 30 min and chased for the indicated times in complete medium (Dulbecco's modified Eagle's medium plus 2% fetal calf serum) alone (0 ng/ml) or complete medium supplemented with TNFα (20 ng/ml; Amersham Pharmacia Biotech) or 50 ng/ml recombinant IL-1α (Amersham Pharmacia Biotech) with 0.5% deoxycholate and 0.1% SDS (radioimmune precipitation buffer). HA-p105 was isolated by immunoprecipitation with 12CA5 mAb, and labeled bands were quantified as for 3T3 cells.

A two-tailed Student’s t test was used to determine whether IKK2 over-expression (3T3 cells) or TNFα stimulation (HeLa stables) induced significant increases in p105 proteolysis compared with the appropriate control cells. The half-life of p105 determined in pulse-chase metabolic labeling experiments was calculated from an extrapolated trend line of mean values (n = 3) using the Microsoft Excel program.

Analysis of p105 Phosphorylation—To analyze in vivo phosphorylation of p105 on serine 927 after cytokine stimulation, 5 × 105 HeLa cells were plated per 100-mm dish. After 18 h in culture, cells were pretreated with 20 μM MG132 proteasome inhibitor (Biomol Research Laboratories) for 30 min and then stimulated for 15 min with TNFα (20 ng/ml; Amersham Pharmacia Biotech) or 50 ng/ml recombinant IL-1α (Amersham Pharmacia Biotech) with 0.5% deoxycholate and 0.1% SDS (radioimmune precipitation buffer). HA-p105 was re-immunoprecipitated with anti-HA mAb. After extensive washing, immunoprecipitates were resuspended in 100 μl of buffer A supplemented with 1% SDS and heated to 100 °C for 5 min. The supernatant was then aspirated and diluted to 1.5 ml in radioimmune precipitation buffer. HA-p105 was re-immunoprecipitated with anti-HA mAb and Western blotted with anti-phospho-Ser927, p105 antibody. The two-step immunoprecipitation procedure disrupted the interaction of transfected HA-p105 with endogenous p105. Analysis of serine 927 phosphorylation of HA-p105 in transiently transfected 3T3 cells was carried out as described previously (3).

For in vitro phosphorylation experiments, wild type or point-mutated HA-p105 was transiently expressed in 293 cells and isolated by immunoprecipitation (26). Immunoprecipitates were washed four times in buffer A and kinase buffer (25 mM Tris, pH 7.5, 5 μM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). Immunoprecipitates were then incubated at room temperature for 30 min in 50 μl of kinase buffer plus 100 μM ATP plus or minus 100 ng of recombinant His6-IKK1 or His6-IKK2 protein (3). The reaction was stopped when immunoprecipitates were washed twice with buffer A. Labeled HA-p105 was eluted by boiling in 100 μl of Laemmli sample buffer and then Western blotted with anti-phospho-Ser927, p105 antibody.

**RESULTS AND DISCUSSION**

**IKK1 and IKK2 Interact with NF-κB1 p105 in Transiently Transfected 293 Cells**—It has recently been reported that IKK2 can form a complex with NF-κB1 p105 in co-transfected cells, although the functional consequence of this interaction was not investigated (14). In initial experiments to analyze further the association of p105 with the IKK complex, 293 cells were transiently transfected with expression vectors encoding HA-p105(S927A) and FL-IKK1, FL-IKK2, or control empty vector (EV). HA-p105/S927A lacks a critical regulatory phosphorylation site involved in signal-induced p105 proteolysis; therefore, its expression levels are not affected when it is co-expressed with active IKK subunits (3). HA-p105(S927A) was directly immunoprecipitated with anti-HA mAb, and co-precipitation of IKK subunits was determined by Western blotting. Both FL-IKK1 and FL-IKK2 specifically co-immunoprecipitated with HA-p105(S927A) at similar levels, confirming previously published data (14, 16) (Fig. 1A). Kinase-inactive (KD) point mutants of FL-IKK1 and FL-IKK2 interacted with HA-p105(S927A) to a similar extent as the wild type molecules (Fig. 1A). In contrast, no association was detected between Myc-p105 and HA-NEMO (Fig. 1B). Therefore, interaction of...
FIG. 1. Interaction of IKK1 and IKK2 with p105. A, 293 cells were co-transfected with the indicated expression vectors and cultured for 36 h. HA-p105(S927A) was immunoprecipitated (Ip) from cell lysates with anti-HA mAb and Western blotted. Blots were sequentially probed with the indicated antibodies. B, HA-NEMO was immunoprecipitated from lysates of 293 cells as in A, and Western blots were probed sequentially with anti-Myc and anti-HA mAbs. C, HeLa cells were pretreated for 30 min with MG132 to block proteasome function. Cells were then cultured for 5 and 15 min with TNFα or control medium before lysis. The IKK complex was isolated from cell lysates by immunoprecipitation with anti-IKK1/2 antiserum. Nonimmune rabbit serum was then cultured for 5 and 15 min with TNFα, and this interaction is not substantially affected by TNFα. Western blots of immunoprecipitated protein was sequentially probed for p105 and NRS (nonprecipitation with anti-IKK1/2 antiserum). Nonimmune rabbit serum was used for control immunoprecipitations. A Western blot of immunoprecipitated protein was sequentially probed for p105 and IKK1/2.

FL-IKK1 and FL-IKK2 with HA-p105 is not mediated via complexing with endogenous NEMO.

To determine whether p105 binds to the IKK complex at physiological expression levels, the endogenous IKK complex was immunoprecipitated with anti-IKK1/2 antibody from HeLaOhio cells pretreated with MG132 inhibitor to block proteasome activity. Low levels of p105 specifically co-immunoprecipitated with IKK1/2 from both control cells and cells stimulated for 5 and 15 min with TNFα (Fig. 1C). Similar results were obtained with cells cultured in the absence of MG132 (data not shown). Thus, the endogenous IKK complex constitutively associates at low stoichiometry with endogenous p105, and this interaction is not substantially affected by TNFα stimulation over a period of 15 min during which maximal p105 Ser927 phosphorylation occurs (3). Similar results were obtained in 293 cells and THP-1 monocytes (data not shown).

To define the sites of interaction for FL-IKK1 and FL-IKK2 on p105, two HA-p105 mutants were generated in which the C terminus was deleted to remove the PEST region or PEST plus DD (see Fig. 2A). These mutants were then transiently expressed in 293 cells with kinase-inactive FL-IKK1 or FL-IKK2, which do not affect HA-p105 expression levels and interact with HA-p105(S927A), similar to their wild type counterparts (Fig. 1A). Removal of the PEST region (HA-p105DE), which contains the IKK target site (3), had little effect on the amount of FL-IKK1 immunoprecipitating with HA-p105 (Fig. 2B). There was a small reduction in the interaction of FL-IKK2 with HA-p105 compared with wild type HA-p105 (Fig. 2B). However, further deletion to remove the DD of FL-IKK2 to control levels (Fig. 2B). A p105 mutant containing an internal deletion (residues 816–892) that specifically removed the DD, HA-p105DD, was also severely impaired in its ability to bind to both FL-IKK1 and FL-IKK2 compared with wild type HA-p105. It was not possible to test whether the p105 DD was sufficient for IKK binding, since the p105 DD (FL-p105DE) was not expressed at significant levels in 293 cells (data not shown). Nevertheless, the data in this section demonstrate that the p105 DD is required for IKK binding to p105, consistent with a recent study by Heissmeyer et al. (14). The PEST region contributes to binding to a more limited extent (Fig. 2B) and probably accounts for the low level of FL-IKK2 binding to HA-p105DD. This may be due to the interaction of the IKK2 kinase domain with its target site on p105 in the 293 over-expression system (16).

It was important to rule out the possibility that DD deletion mutants failed to bind IKK1 and IKK2 due to gross structural alterations in p105. The DD structure of Fas and other DDs comprises six anti-parallel, amphipathic α-helices (21, 29). A
triggers apoptosis (29). To determine whether signaling intermediate FADD and therefore cannot regions of the DD (31). V238N Fas cannot bind to its down-protein with no detectable conformational changes in other regions of the DD (31). V238N in Fas was mutated in p105 to generate the V238N in Fas was mutated in p105 to generate the ft-helix 3 of the wild type protein with no detectable conformational changes in other regions of the DD (31). V238N Fas cannot bind to its downstream signaling intermediate FADD and therefore cannot trigger apoptosis (29). To determine whether d-helix 3 in the p105 DD is involved in binding to IKK, the residue corresponding to V238N in Fas was mutated in p105 to generate the HA-p105(L841A) mutant (see Fig. 2A). This mutant was also impaired in its ability to bind to both FL-IKK1 (KD) and FL-IKK2 (KD) compared with wild type HA-p105 when over-expressed in 293 cells, although not to such a great extent as HA-p105(ΔDD) (Fig. 2C). These data indicate that d-helix 3 of p105 DD is involved in binding to the IKK complex and are consistent with the demonstration by Heissmeyer et al. that the IKK2 binding site resides in the N-terminal half of the p105 DD (14). The reduced binding of HA-p105(L841A) to IKK1 and IKK2 also suggests that gross conformational changes do not account for the inability of DD deletion mutants of HA-p105 to bind FL-IKK1/2. Consistent with this hypothesis, all of the p105 deletion mutants could bind to RelA with equal efficiency (Fig. 3A). In addition, the p105 deletion mutants exclusively localized to the cytoplasm similar to the WT (Fig. 3B). Thus, deletion of the PEST region and/or DD did not affect the function of ankyrin repeats of p105, as reported previously (33).

A Functional p105 DD Is Required for Efficient Serine 927 Phosphorylation in Vitro by IKK1 or IKK2—In many cases, the specificity of protein kinases is determined by docking sites within the substrate, distinct from the phosphoacceptor site (34). Such docking sites recruit kinases to their correct substrates and enhance their fidelity and efficiency of action. Serine 927 is essential for signal-induced proteolysis of p105 and is directly phosphorylated by purified IKK1 and IKK2 in vitro (3). In initial experiments, therefore, the role of the DD in phosphorylation of p105 serine 927 by IKK in vitro was investigated. To do this, HA-p105, HA-p105(ΔDD) or HA-p105(L841A) was transiently expressed in 293 cells and isolated by immunoprecipitation with anti-HA mAb. The immunoprecipitated proteins were then phosphorylated by recombinant IKK1 or IKK2 protein and Western blotted with anti-phospho-Ser927-p105 antibody (Fig. 4, A and B). Both IKK1 and IKK2 clearly phosphorylated serine 927 of wild type HA-p105. In contrast, IKK1 and IKK2 induced little detectable serine 927 phosphorylation of either HA-p105(ΔDD) or HA-p105(L841A). Immunoblotting with anti-p105 antibody confirmed that similar levels of HA-p105 were immunoprecipitated in each case. These experiments indicate that the DD is essential for both IKK1 and IKK2 to efficiently phosphorylate HA-p105 on serine 927 in vitro.

Efficient p105 Degradation Induced by Over-expressed IKK2 Requires a Functional p105 DD—This laboratory has previously shown that FL-IKK2 triggers degradation of HA-p105 when co-expressed in 3T3 cells (3). Therefore, to determine whether the p105 DD is important for the IKK complex to regulate p105 proteolysis in vivo, an expression vector encoding FL-IKK2 or an empty vector with no insert (EV) was co-transfected in 3T3 cells with vectors encoding wild type, ΔDD or L841A HA-p105. HA-p105 turnover was then determined in pulse-chase metabolic labeling experiments. The half-life of

FIG. 3. All p105 mutants bind to RelA and are retained in the cytoplasm. A, 293 cells were co-transfected with vectors encoding RelA and wild type, deletion, or point mutants of HA-p105. Lysates were prepared from cells after 36 h of culture and immunoprecipitated with anti-HA mAb. Western blots of immunoprecipitated protein (Lysate Ip HA) and total cell lysates (Lysate) were probed with the antibodies shown. B, 293 cells were transfected with vectors encoding the indicated HA-p105 constructs. Nuclear (N) and cytoplasmic (C) fractions were prepared and Western blotted sequentially with the antibodies shown.

FIG. 4. Phosphorylation of p105 in vitro on serine 927 by IKK1 and IKK2 requires a functional p105 death domain. A and B, 293 cells were transfected with HA-p105, HA-p105(ΔDD), or HA-p105(L841A). Lysates were prepared after 24 h in culture and immunoprecipitated with anti-HA mAb. Immunoprecipitates were phosphorylated in vitro with IKK1 or IKK2 and then Western blotted. Blots were sequentially probed with anti-phospho-Ser927-p105 and anti-p105 antibodies.
wild type HA-p105 (mean $t_{1/2} = 9.7$ h) was dramatically reduced by FL-IKK2 co-expression (mean $t_{1/2} = 2.5$ h) as previously shown (Fig. 5A; Ref. 3). Analysis of the 4-h point confirmed that the reduction in remaining HA-p105 induced by FL-IKK2 co-expression was significant ($p = 0.001; n = 3$). However, FL-IKK2 co-expression had little effect on the turnover of HA-p105(ΔDD) (mean $t_{1/2}$ with FL-IKK2 was 9.9 h) or HA-p105(L841A) (mean $t_{1/2}$ with FL-IKK2 was 9.5 h). Consistent with this, there was no significant difference in the amount of HA-p105 remaining with or without FL-IKK2 at 4 h for either HA-p105(ΔDD) ($p = 0.054; n = 3$) or HA-p105(L841A) ($p = 1.0; n = 3$). Thus, the DD is required for over-expressed FL-IKK2 to efficiently induce HA-p105 proteolysis.

FL-IKK2 triggers degradation of HA-p105 by phosphorylating serine 927 in the p105 PEST region (3). The role of the DD in FL-IKK2-mediated phosphorylation of serine 927 was determined by co-transfection of 3T3 cells and Western blotting of anti-HA immunoprecipitates with anti-Ser927-p105 antibody. FL-IKK2 co-expression clearly induced phosphorylation of wild type HA-p105. However, FL-IKK2 co-expression did not induce detectable phosphorylation of either HA-p105(ΔDD) or HA-p105(L841A), although similar amounts of HA-p105 were immunoprecipitated in each case (Fig. 5B), and FL-IKK2 was expressed at the same level for each transfection (Fig. 5B, lower panel). The DD of p105 is therefore essential for its efficient phosphorylation on serine 927 by FL-IKK2 in vivo, explaining why the half-life of HA-p105 DD mutants is unaffected when co-expressed with FL-IKK2 (Fig. 5A).

The p105 DD Is Not Required for HA-p105 Proteolysis Induced by High Concentrations of FL-IKK2—The previous data were consistent with the hypothesis that the p105 DD of p105 functions as a docking site for IKK1 and IKK2. The DD therefore may increase the local concentration of IKK on p105, facilitating serine 927 phosphorylation. This model predicts that high concentrations of transfected FL-IKK2 might overcome the requirement for the p105 DD to trigger phosphorylation of HA-p105 on serine 927. Accordingly, FL-IKK2 was expressed at high and low concentrations together with wild type and DD mutants of HA-p105. 293 cells were used for this experiment, since much higher levels of transiently expressed protein can be obtained than is possible in 3T3 fibroblasts. HA-p105 was immunoprecipitated, and phosphorylation of serine 927 was determined by Western blotting. When expressed at a low concentration (20 ng of plasmid), FL-IKK2 clearly induced serine 927 phosphorylation of wild type HA-p105 but not of either HA-p105(ΔDD) or HA-p105(L841A) (Fig. 5C). Similar levels of HA-p105 were immunoprecipitated in each case. However, at the high FL-IKK2 concentration (500 ng of plasmid), all three HA-p105 proteins were phosphorylated on serine 927. Thus, high concentrations of FL-IKK2 induce p105 serine 927 phosphorylation independently of the p105 DD. Binding to the p105 DD, therefore, appears to increase the effective concentration of IKK available to phosphorylate p105, thereby increasing its efficiency of action.

p105 DD Is Essential for TNFα-induced Degradation of p105—It was important to demonstrate that the p105 DD is required for proteolysis of p105 induced by physiological stimulation with a cytokine, which involves p105 phosphorylation by the endogenous IKK complex, in addition to its important role in proteolysis triggered by IKK2 over-expression. To this end, HeLa cells were transfected with plasmids encoding HA-p105, HA-p105(ΔDD) or HA-p105(L841A) and stable clones isolated after G418 selection. Pulse-chase experiments were then carried out using clones with expression levels similar to endogenous p105 (Fig. 6A). TNFα increased the rate of turnover of wild type HA-p105 (mean $t_{1/2}$ without TNFα was 5.5 h; mean $t_{1/2}$ with TNFα was 0.9 h) as expected (3). At 90 min, the amount of HA-p105 remaining was significantly reduced with
TNFα (p = 0.0004; n = 3). In contrast, TNFα stimulation did not alter the turnover of HA-p105(ΔDD) (mean t1/2 with TNFα was 4.6 h), and at 90 min there was no significant difference in the amount of HA-p105(ΔDD) remaining with or without TNFα (p = 0.225; n = 3). Similarly, TNFα stimulation did not induce proteolysis of HA-p105(L841A) or a significant reduction in the amount of HA-p105(L841A) remaining after a 90-min chase (p = 0.604; n = 3). However, the basal turnover of HA-p105(L841A) was consistently increased relative to wild type HA-p105 (mean t1/2 = 1.1 h). The reason for this increase is unclear. Control experiments demonstrated that IκBα was similarly degraded following TNFα stimulation in all three clones (data not shown). The TNFα signaling pathway controlling the IKK complex was therefore intact in both the HA-p105(ΔDD) and HA-p105(L841A) clones.

The ability of TNFα to induce serine 927 phosphorylation in each of the clones was also determined by anti-HA mAb immunoprecipitation and Western blotting (Fig. 6B). Serine 927 phosphorylation of HA-p105 was clearly induced after 15 min of TNFα stimulation. However, TNFα-induced serine 927 phosphorylation was completely blocked for both HA-p105(ΔDD) or HA-p105(L841A).

Together, these data demonstrate that the p105 DD is absolutely required for TNFα-induced serine 927 phosphorylation and subsequent p105 degradation, confirming the importance of the p105 DD in regulating signal-induced p105 proteolysis. Since IKK-mediated phosphorylation of p105 is essential for cytokine-induced p105 degradation (9), this is likely to reflect the role of the p105 DD in facilitating IKK-mediated phosphorylation of p105 serine 927.

The p105 DD therefore increases the efficiency with which IKK can phosphorylate serine 927 in the p105 PEST region in vivo and trigger p105 degradation. This presumably results from the increase in local concentration of IKK2 in the immediate vicinity of the target phosphorylation sequence on p105 mediated by recruitment via the p105 DD. The preassembly of IKK with p105 may increase its specificity for this substrate and also shorten the time between kinase activation and serine 927 phosphorylation (34). The IKK complex has also been shown to bind to IκBα in a NEMO-dependent fashion (35), and this interaction facilitates IKK phosphorylation of IκBα.

In future studies, it will be important to determine whether any of the signaling intermediates upstream of the IKK complex are distinct for the regulation of IκBα and p105.

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