The Non-ankyrin C Terminus of IκBα Physically Interacts with p53 in Vivo and Dissociates in Response to Apoptotic Stress, Hypoxia, DNA Damage, and Transforming Growth Factor-β1-mediated Growth Suppression*

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Transforming growth factor beta (TGF-β1) suppresses the growth of mink lung Mv1Lu epithelial cells, whereas testicular hyaluronidase abolishes the growth inhibition. Exposure of Mv1Lu cells to TGF-β rapidly resulted in down-regulation of cytosolic IκBα and hyaluronidase prevented this effect, suggesting a possible role of IκBα in the growth regulation. Ectopic expression of wild-type and dominant negative IκBα prevented TGF-β1-mediated growth suppression. Nonetheless, the blocking effect of IκBα is not related to regulation of NF-κB function by its N-terminal ankyrin-repeat region (amino acids 1–243). Removal of the PEST (proline-glutamic acid-serine-threonine) domain-containing C terminus (amino acids 244–314) abolished the IκBα function, and the C terminus alone blocked the TGF-β1 growth-inhibitory effect. Co-immunoprecipitation by anti-p53 antibody using Mv1Lu and other types of cells, as well as rat liver and spleen, revealed that a portion of cytosolic IκBα physically interacted with p53. In contrast, Mdm2, an inhibitor of p53, was barely detectable in the immunoprecipitates. The cytosolic p53-IκBα complex rapidly dissociated in response to apoptotic stress, etoposide- and UV-mediated DNA damage, hypoxia, and TGF-β1-mediated growth suppression. Also, a rapid increase in the formation of the nuclear p53-IκBα complex was observed during exposure to etoposide and UV. In contrast, TGF-β1-mediated promotion of fibroblast growth failed to mediate p53-IκBα dissociation. Mapping by yeast two-hybrid showed that the non-ankyrin C terminus of IκBα physically interacted with the proline-rich region and a phosphorylation site, serine 46, in p53. Deletion of serine 46 or alteration of serine 46 to glycine abolished the p53-IκBα interaction. Alteration to threonine retained the binding interaction, suggesting that serine 46 phosphorylation is involved in the p53-IκBα complex formation. Functionally, enhancement of p53 apoptosis was observed when p53 and IκBα were transiently co-expressed in cells. Together, the IκBα-p53 complex plays an important role in responses involving growth regulation, apoptosis, and hypoxic stress.

Transforming growth factor beta (TGF-β) plays an important role in controlling embryogenesis, immune cell functions, and extracellular matrix homeostasis (Refs. 1–4; for reviews). TGF-β promotes the growth of fibroblasts and other cell types, whereas it inhibits epithelial cell growth and induces apoptosis of hepatocytes and various cancer cells. The underlying mechanisms are unknown.

TGF-β signaling involves binding of TGF-β to the type II receptor, followed by recruiting the type I receptor, then inducing phosphorylation and hetero-dimerization of Smad2 and 3, and binding of Smad4 to the Smad2/3 complex. The Smad2/3/4 protein complex migrates to the nucleus and regulates the transcription of target genes (1, 2).

Cross-talk of the TGF-β/Smad pathway with other cellular signaling pathways has been demonstrated (5). For example, the Smad2/3/4 protein complex may recruit c-Jun, ATF-2, CBP/p300, and STAT3 in the transcriptional regulation of a target gene (2). These recruited proteins are also transducers of the stress and STAT signaling pathways, respectively. NF-κB p65 regulates Smad7 promoter activity (6). Smad7 is an inhibitor of the TGF-β signaling pathway. TGF-β activates Ras and three members of the mitogen-activated protein kinase (MAPK) superfamily in epithelial cells (7). However, TGF-β-mediated activation of p44/42 MAPK (ERK) pathway is due to autocrine secretion of basic fibroblast growth factor in fibroblasts (8).

TGF-β1 and its induced matrix protein(s) protect L929 fibroblasts from killing by tumor necrosis factor (TNF) (9–11). This protective function is effectively abolished by testicular hyaluronidase (12, 13). TGF-β1 suppresses the proliferation of mink lung epithelial Mv1Lu cells and hyaluronidase inhibits the suppression (12, 13). Here, it is demonstrated that TGF-β1 rapidly down-regulated IκBα expression in Mv1Lu cells and hyaluronidase abolished the down-regulation, suggesting that IκBα participates in the TGF-β1-mediated growth suppression. Ectopic expression of IκBα blocked TGF-β1-mediated growth suppression of Mv1Lu cells, and the non-ankyrin C terminus of IκBα was found to contribute to this regulatory activity. This region of IκBα contains an acidic PEST (proline-glutamic acid-serine-threonine) domain and fails to inhibit NF-κB function (14).

Notably, a portion of cytosolic IκBα was found to interact...
p53 and IκBα Binding Interaction

TABLE I

| PCR primers and expression constructs |
|---------------------------------------|

| Constructs                      | PCR primers (5' to 3') |
|----------------------------------|-----------------------|
| pEGFP-C1 (N-terminal GFP tag; EcoR1) | Forward: TCGAATTTCTAGTTTCCAGCAGGCTGGG |
| 1. IκBα (1–314)                  | Reverse: GCAGAATTCTTTAATGTCAGACCGTGG |
| 2. IκBα243N (1–243)               | Reverse: GCAGAATTCTTTAATGTCAGACCGTGG |
| 3. IκBα243C (244–314)             | Reverse: GCAGAATTCTTTAATGTCAGACCGTGG |

| pSos (N-terminal Sos tag; MluI)   | PCR primers (5' to 3') |
|----------------------------------|-----------------------|
| 1. IκBα (1–314)                  | Forward: GCCACCGTGATGGTTTCCAGCAGGCTGGG |
| 2. IκBα243N (1–243)               | Reverse: CACCGTGCGTATTAAATGTCAGACCGTGG |
| 3. IκBα243C (244–314)             | Reverse: CACCGTGCGTATTAAATGTCAGACCGTGG |

| pDsRed-N1 (C-terminal DsRed tag; EcoR1) | PCR primers (5' to 3') |
|----------------------------------------|-----------------------|
| 1. IκBα (1–393)                       | Forward: TCGAATTTCTAGTTTCCAGCAGGCTGGG |
| 2. IκBα243N (1–243)                   | Reverse: GCAGAATTCTTTAATGTCAGACCGTGG |
| 3. IκBα243C (244–314)                 | Reverse: GCAGAATTCTTTAATGTCAGACCGTGG |

| pcDNA3.1.TOPO (TA cloning)            | PCR primers (5' to 3') |
|---------------------------------------|-----------------------|
| 1. FLAG-p53 (1–393)                   | Forward: ACCATGGGATTACAAGGAGGACGCCAGGTC |
| 2. IκBα243N (1–243)                   | Reverse: CACGCGTGCGTTATAATGTCAGACCGTGG |
| 3. IκBα243C (244–314)                 | Reverse: CACGCGTGCGTTATAATGTCAGACCGTGG |

| Mutant constructs by site-directed mutagenesis | PCR primers (5' to 3') |
|-----------------------------------------------|-----------------------|
| pMyr (N-terminal Myr tag)                     | PCR primers (5' to 3') |
| 1. p53Δ46                                      | Forward: GATGATTTTGATGTTCCG GCCAGCAGTATTGAA |
| 2. p53S46G                                    | Reverse: TTCAATATCGTCCGGCAGCATCAAATCACAT |
| 3. p53S64T                                    | Reverse: ATGATTTTGATGTTCCG GCCAGCAGTATTGGA |
|                                               | Reverse: TCAATATCGTCCGGCAGCATCAAATCACAT |

* The numbers in parentheses are the amino acid numbers.

with p53 in Mv1Lu and other unstimulated resting cells, as well as rat liver and spleen. Dissociation of the p53-IκBα complex occurred in response to growth suppression by TGF-β1, apoptotic stress, hypoxia, DNA damage, and UV radiation. The dissociation allows p53 nuclear translocation. In contrast, TGF-β1-mediated promotion of fibroblast growth failed to mediate p53-IκBα dissociation. Enhancement of p53 apoptosis was observed when various cells were co-transfected with p53 and IκBα expression constructs. This study suggests that IκBα regulates cytosolic p53 function in resting cells and participates in p53-mediated apoptosis in response to various stresses. The structural basis of the p53-IκBα binding interaction was analyzed by the yeast two-hybrid system, and functional significance of this binding interaction was discussed.

EXPERIMENTAL PROCEDURES

Cell Lines—Mink lung Mv1Lu epithelial cells, human Molt-4 T cells, human prostate Du145 cells, monkey kidney Cos7 fibroblasts, and murine L929 fibroblasts were from American Type Culture Collections (ATCC, Manassas, VA) and cultured according to provider’s instructions. Human SK-SH cell lines, human ovarian HeLa cells, and human breast cancer MCF-7 cells were kindly provided by Jeff Mattison and Dr. John Noti of this Institute, respectively. A L929 cell line, which stably expressed GFP, was established as previously described (9).

Chemicals and Proteins—Recombinant human TNF-α (or TNF) was from Genzyme (Boston, MA) and R&D (Minneapolis, MN). Purified human platelet TGF-β1 was from R&D. Polyclonal antibodies against p53, p65 NF-κB, Mdm2, FLAG epitope, and IκBα were from Santa Cruz Biotechnology (Santa Cruz, CA) and BD PharMingen (San Diego, CA). Also, agarose bead-conjugated IgG against p53 and IκBα were from Santa Cruz Biotechnology.

DNA Constructs—A murine full-length IκBα cDNA was found in the universal EST data base (GenBank accession AA606238) and the IκBα cDNA was found in the universal EST data base (GenBank accession AA606238) and the clone was obtained from the Incyte Genosystems (St. Louis, MO). Three expression constructs were made with the pEGFP-C1 vector (cloning

![FIG. 1. Hyaluronidase counteracts TGF-β1-mediated growth suppression of Mv1Lu cells.](image)
FIG. 2. TGF-β1 down-regulates IκBα, and hyaluronidase blocks the down-regulation in Mv1Lu cells. A, Mv1Lu cells were pretreated with or without hyaluronidase (100 units/ml) for 1 h, followed by exposure to TGF-β1 (2 ng/ml) for 0.5–4 h. The expression of cytosolic p65 NF-κB and IκBα in these cells was examined by Western blotting. TGF-β1 rapidly down-regulated IκBα, whereas hyaluronidase blocked the down-regulation. The cytosolic levels of p65 NF-κB were not affected by TGF-β1 and/or hyaluronidase. B, in comparison, L929 fibroblasts were pretreated with hyaluronidase (100 units/ml) for 1 h and then co-treated with TGF-β1 (2 ng/ml) for various indicated times. The cytosolic level of IκBα was not affected by TGF-β1 and/or hyaluronidase. TGF-β1 reduced the cytosolic level of p65 NF-κB in a time-related manner. Hyaluronidase-pretreated cells had a reduced level of cytosolic p65 NF-κB, whereas TGF-β1 restored the NF-κB level.

FIG. 3. The non-ankyrin C terminus of IκBα inhibits TGF-β1-mediated growth of Mv1Lu cells. Mv1Lu cells were transfected with various GFP-tagged IκBα constructs by electroporation and cultured in 30-mm Petri dishes overnight, and expression of these GFP-tagged proteins was examined by fluorescence microscopy. The cells were than exposed to TGF-β1 for 48 h, followed by staining with crystal violet to determine the extent of growth inhibition. Shown in the graph are representative data from three experiments. A schematic diagram of IκBα is also shown. The N terminus (amino acids 1–68) contains two ubiquitination sites, two serine phosphorylation sites (Ser-32 and Ser-36) and five ankyrin repeats at the central region, followed by a PEST domain-containing C terminus (amino acids 244–314). GFP, control pEGFP-C1 construct; GFP-IκBα, GFP-tagged wild-type IκBα; GFP-DN-IκBα, GFP-tagged dominant negative IκBα (S32R and S36A); GFP-IκBα243N, GFP-tagged IκBα N terminus (amino acids 1–243); GFP-IκBα243C, GFP-tagged IκBα C terminus (amino acids 244–314).

site, EcoRI; CLONTECH, Palo Alto, CA): (i) IκBα, the entire coding region (amino acids 1–314); (ii) IκBα243N, the N-terminal surface-exposed and the ankyrin region (amino acids 1–243); (iii) IκBα243C, the C-terminal acidic PEST domain region (amino acids 244–314) (see Fig. 3). Additionally, these IκBα cDNAs were constructed with the pSos vector (cloning site, MLu1; Stratagene, San Diego, CA) for yeast two-hybrid experiments as described below. Wild-type p53 cDNA (18) was constructed in pDsRed-N1 vector (CLONTECH) and tagged with a C-terminal red fluorescence protein. Also, p53 was tagged with an N-terminal FLAG epitope using pcDNA3.1 vector (Invitrogen). Shown in the Table I are the synthetic primers used for making these constructs by PCR.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, LaJolla, CA). A dominant negative IκBα construct was made by altering Ser-32 to Arg-32 and Ser-36 to Ala-36 (19) using the following primers: forward, 5′-CGGCCACGACAGAGGCCTGGACGCCATGAAGGA-3′; reverse, 5′-TCCCTCTAGGGCCTGCCAGTCTGTTGGCCGGC-3′. Electroporation—Mv1Lu cells (3 million cells in 500 μl of serum-free minimal medium) were electroporated in duplicates with 20 μg of the above expression constructs (200 V and 50 ms using the BTX ECM 630 electroporator, Genetronics, San Diego, CA). The cells were then seeded onto 30-mm Petri dishes, grown overnight, and exposed to TGF-β1 (2 ng/ml) for 48 h. The cells were then stained with crystal violet to determine the extent of cell growth as well as growth inhibition by TGF-β1. In other experiments, Mv1Lu and other types of cells were co-transfected with p53-ΔsRed and the full-length IκBα or IκBα243C (in pEGFP-C1) by electroporation. The cells were cultured for 48 h, and the extent of p53-mediated cell death was examined by crystal violet staining.

Yeast Two-hybrid Interactions—To determine whether p53 physically interacts with IκBα in vivo, we utilized the CytoTrap yeast two-hybrid system (Stratagene) as previously described (18). Binding of a cytosolic Sos-tagged bait protein to a cell membrane-anchored target protein (tagged with a myristoylation signal) results in activation of the
Ras signaling pathway in yeast. This binding interaction allows mutant yeast cdc25H to grow in 37 °C using a selective agaro plate containing galactose. Without binding, yeast cells fail to grow at 37 °C. The above-indicated IxBo constructs were used as baits (in pSos vector; see Table 1 for synthetic primers). The following pS3 target constructs were made as previously described (18): (i) a full-length pS3, (ii) a partial N-terminal pS3 (amino acids 1–100), and (iii) the proline-rich (or growth regulatory) region of pS3 (amino acids 66–100). Additionally, a pS3 construct without the proline-rich region was made (in pMyr vector). The original proline-rich region-deleted pS3 construct (20) was a gift of Dr. U. Moll of the State University of New York at Stony Brook and Dr. A. J. Levine of the Rockefeller University. Self-interaction between pSos-MafB and pMyr-MafB as well as pSos-p53 and pSos-WOX1 (WW domain oxidoreductase) (18) were tested as positive controls. Vector combinations for negative binding interactions were empty pSos versus empty pMyr and pSos-Lamin C versus pMyr-p53.

Immunoprecipitation—Immunoprecipitation was performed as previously described (18). Both cytosolic and nuclear proteins from Mv1Lu, L929, and other types of cells were extracted using the NE-PER Nuclear and Cytoplasmic Extraction reagents (Pierce, Rockford, IL). Endogenous pS3 was precipitated by antibodies against pS3 and protein A-agarose (Pierce), separated by SDS-PAGE, and detected in Western blotting. Co-precipitation of IxBo with pS3 was determined using anti-IxBo antibody in duplicate precipitation experiments. In additional experiments, FLAG-tagged pS3 (in pcDNA3.1) was expressed in cells and precipitated by anti-FLAG antibody (Santa Cruz Biotechnology) from the cell lysates. The presence of FLAG-pS3 and the co-precipitated endogenous IxBo was determined by antibodies against pS3 and IxBo, respectively, in Western blotting. Where indicated, agarose bead-conjugated anti-pS3 IgG was used in immunoprecipitation experiments. The immobilized anti-pS3 IgG was less effective in binding to antigen than the solution-phase anti-pS3 IgG.

Additionally, liver extracts from male Wistar rats were prepared by an ultrasonication-based Tissumizer (Tekmar, Cincinnati, OH), using a lysis buffer containing a mixture of protease inhibitors (11). The cytosolic lysate (~5 mg) was incubated with agaro beads covalently conjugated with anti-IxBo IgG or anti-pS3 IgG (Santa Cruz Biotechnology) and rotated at 4 °C for 4 h. Co-precipitation of IxBo and pS3 was then determined in Western blotting.

Transient Expression—Where indicated, Mv1Lu cells were cultured on coverslips overnight and co-transfected with IxBo-pEGFP-C1 and pS3-DeRed constructs by FuGENE (Roche Molecular Biochemicals, Indianapolis, IN), a liposome-based reagent. Twenty-four hours later, the cells were fixed with 3.3% formaldehyde and permeabilized with 0.1% Triton X-100 (Sigma) and stained with the nuclear dye DAPI (Calbiochem). The cells were examined under fluorescence microscopy.

Apoptosis, Hypoxia, DNA Damage, and UV Radiation—L929 and other indicated cells were treated with staurosporine (1 μM) for 1 h to induce apoptosis, followed by preparation of cytosolic and nuclear fractions from the cells and immunoprecipitation using anti-pS3 IgG beads (or anti-pS3 IgG and protein A-agarose beads). Similarly, the cells were cultured under hypoxic conditions in the presence of deferoxamine (100 μM) for 16 h prior to processing cell lysis and immunoprecipitation. Also, cells were treated with etoposide (50 μg/ml) or exposed to UV light (120 mj/cm2; using a UV cross-linker from Fisher Scientific). One hour later, the cells were subjected to lysis and co-immunoprecipitation. Where indicated, fresh spleen cells were isolated from the spleens of sacrificed male Wistar rats and treated similarly as above.

RESULTS

TGF-β1-mediated Growth Inhibition of Epithelial Mv1Lu Cells Is Blocked by Hyaluronidase and Is Related with Down-regulation of IxBo—TGF-β1 inhibits the proliferation of Mv1Lu epithelial cells (13). Exposure of Mv1Lu cells to TGF-β1 for 48 h resulted in growth inhibition by ~40% (Fig. 1A). We have shown that bovine testicular hyaluronidase counteracts TGF-β1-mediated protection of TGF-α cytotoxicity in L929 and LNCaP prostate cells (13). In agreement with previous observations (13), treatment of Mv1Lu cells with hyaluronidase resulted in increased cell proliferation and reduction of TGF-β1-mediated growth inhibition in a dose-dependent manner (Fig. 1A).

TGF-α antagonizes various functions of TGF-β1 (9, 11, 12). For example, TGF-β1 protects L929 fibroblasts from TNF-mediated cell death (11). Whether TGF-α blocked the inhibitory effect of TGF-β1 in Mv1Lu cell growth was determined. The results showed that TGF-α failed to prevent TGF-β1-mediated growth inhibition (Fig. 1B). Indeed, both TGF-α and TGF-β1 suppressed the proliferation of Mv1Lu cells in an additive manner (Fig. 1B).

To determine the mechanisms for the functional antagonism between TGF-β1 and hyaluronidase, Mv1Lu cells were treated with TGF-β1 for various indicated times, followed by determining the expression of p65 NF-κB and IxBo by Western blotting.

FIG. 5. TNF-α and staurosporin-mediated dissociation of p53-IxBo complex in L929 and HeLa cells. A, TNF-α mediated IxBo degradation in L929 fibroblasts but not in an established L929 cell line stably expressing GFP (L929-GFP) (left panel). L929-GFP cells were treated with TNF-α (50 ng/ml) for 1 h, followed by preparing cytosolic and nuclear fractions and immunoprecipitation using anti-p53 IgG agarose beads. One-tenth (~20 μg) of the total proteins were loaded for SDS-PAGE, and expression of p53, Mdm2, and IxBo was examined using specific antibodies (right panel; see Fig. 1; Pre-IP). IxBo, but not Mdm2, was co-precipitated with p53 in the cytosol and TNF-α mediated p53-IxBo dissociation (right panel; see IP with anti-p53). B, under similar experimental conditions, L929 cells were treated with staurosporine (1 μM) for 1 h, followed by determining the presence of p53-IxBo complex by co-immunoprecipitation. The p53-IxBo complex was found in the cytosol, and staurosporine mediated the complex dissociation. C, a similar experiment was performed using ovarian HeLa cells. Again, staurosporine mediated the dissociation of cytosolic p53/IxBo complex, along with p53 nuclear translocation.
TGF-β1 rapidly down-regulated the expression of cytosolic IκBα in 30 min, whereas it had no effect on p65 NF-κB (Fig. 2A). Notably, pretreatment of Mv1Lu cells with hyaluronidase for 1 h, followed by exposure to TGF-β1, prevented the down-regulation of IκBα (Fig. 2A). These results suggest that suppression of IκBα expression is necessary for TGF-β1-mediated growth suppression of Mv1Lu cells.

Either TGF-β1 or hyaluronidase increases L929 cell growth by 10–30% in 48 h (11, 13). However, no significant enhancement of cell growth (0 ± 10%) was observed when L929 cells were exposed to both proteins. In control experiments, L929 fibroblasts were pretreated with hyaluronidase for 1 h and then exposed to TGF-β1 for various indicated times. The cytosolic level of IκBα was not affected by TGF-β1 or by TGF-β1 and hyaluronidase in combination (Fig. 2B). TGF-β1 reduced the cytosolic level of p65 NF-κB in a time-related manner (Fig. 2B). Hyaluronidase-pretreated cells had a reduced level of cytosolic p65 NF-κB, whereas TGF-β1 restored the NF-κB level (Fig. 2B).

The Non-ankyrin C Terminus of IκBα Blocks TGF-β1-mediated Growth Inhibition of Mv1Lu Cells—To determine whether IκBα is involved in the TGF-β1-mediated growth suppression, Mv1Lu cells were electroporated with the control GFP or the GFP-IκBα construct. Following overnight culturing, the cells were treated with TGF-β1 for 48 h. TGF-β1 reduced 37.7% of the growth inhibition of GFP-expressing cells, whereas IκBα reduced the growth inhibition down to 10.7% (Fig. 3). Dominant negative IκBα also reduced the growth inhibitory effect of TGF-β1 to 2.3% (Fig. 3).

The dominant negative IκBα could not undergo phosphorylation, ubiquitination, and degradation, thereby effectively preventing activation of NF-κB (19). Nonetheless, blocking of TGF-β1-mediated growth inhibition is not related with IκBα regulation of NF-κB function. The inhibitory effect of IκBα on TGF-β1 was abolished by removing its non-ankyrin C terminus. That is, ectopic expression of IκBα243N, the N-terminal ankyrin-rich or NF-κB-interacting region (amino acids 1–243) (14), in Mv1Lu cells failed to block the growth inhibitory effect of TGF-β1 (52.8% growth inhibition) (Fig. 3). In contrast, the non-ankyrin C terminus, IκBα243C (amino acids 244–314), abolished the TGF-β1 effect (−2.0% growth inhibition) (Fig. 3).

IκBα Physically Interacts with p53 in Vivo—p53 is involved in the TGF-β1-mediated growth suppression and apoptosis (21–24). The possible binding interaction between p53 and IκBα in vivo was examined. Immunoprecipitation of both cytosolic and nuclear p53 from Mv1Lu cells was performed using anti-p53 IgG antibody (against wild-type p53). Endogenous p53 was found in both cytosol and nucleus (Fig. 4A). IκBα was co-precipitated with p53 in the cytosol but not in the nucleus (Fig. 4A). The p53-IκBα interaction could not be abolished by treatment of cells with EDTA (5 mM), indicating that the binding is calcium-independent.

Similarly, transient overexpression of FLAG-tagged p53 in L929 cells, followed by overnight culturing and processing immunoprecipitation using anti-FLAG IgG, resulted in the presence of FLAG-p53 in the cytosol and nucleus (Fig. 4B). Endogenous IκBα was co-precipitated with FLAG-p53 in the cytosol but not in the nucleus (Fig. 4B).

When rat liver cytosolic extract was incubated with immobilized anti-IκBα IgG beads, p53 was found in the precipitate of IκBα (Fig. 4C), again indicating that a portion of cytosolic p53 physically interacts with IκBα in vivo. Similarly, precipitation of p53 using anti-p53 antibody also resulted in the co-presence of p53 and IκBα in the precipitate using the rat liver cytosolic extract (Fig. 4C). In negative controls, when the above antibodies were heat-inactivated (75 °C for 30 min) and used for immunoprecipitation, no precipitated target proteins were found (data not shown).

In parallel with the above observations, co-transfection of Mv1Lu cells with p53-3xGFP and IκBα-pEGFP-C1 constructs was performed. The cells were cultured overnight and then examined under fluorescence microscopy. Both the expressed p53-3xGFP and GFP-IκBα were co-localized in the cytoplasm (Fig. 4D).

Dissociation of p53-IκBα Complex in Response to Apoptotic Stress, Hypoxia, DNA Damage, and TGF-β1-mediated Growth Suppression—To further elucidate the functional significance of p53-IκBα binding in vivo, various cell lines were treated with TGF-β1, TNF, staurosporine, and deoxefaramine, then the dissociation of p53-IκBα complex in the cytoplasm and nucleus was examined.

Exposure of L929 cells to TNFα resulted in IκBα degradation (Fig. 5A). Interestingly, the TNF-dependent IκBα degradation was abolished in an established L929 cell line stably expressing GFP (Fig. 5A). This cell line is suitable for examining TNF-mediated p53-IκBα complex formation and dissociation. The abundance of cytosolic IκBα was greater than that of p53 (Fig. 5A; see Pre-IP). Mdm2, a known inhibitor of p53, was barely detectable (Fig. 5A). Indeed, p53 level is normally low in unstimulated resting cells (Ref. 25, review). Precipitation of p53 with anti-p53 IgG beads showed the presence of cytosolic p53 and IκBα in the precipitate, and TNF mediated p53-IκBα dissociation (Fig. 5A). Mdm2 was barely detectable (Fig. 5A). The results indicate that the cytosolic p53 complexes with IκBα, rather than with Mdm2, in unstimulated L929 cells. Also, only a small portion of cytosolic IκBα physically interacts with p53.

Similarly, staurosporine-mediated apoptosis of L929 cells also resulted in p53-IκBα dissociation in the cytosol (Fig. 5B).
No IκBα degradation was observed in the staurosporine-treated L929 cells (see Pre-IP, Fig. 5B). Similarly, cytosolic p53-IκBα dissociation, along with p53 nuclear translocation, was observed in the staurosporine-treated ovarian HeLa cells (Fig. 5C).

In contrast to the above-indicated cells, there was an increased amount of IκBα associated with p53 in the cytoplasm and nucleus of unstimulated neuronal SK-N-SH cells (Fig. 6A). Staurosporine mediated p53-IκBα dissociation but could not induce IκBα degradation (Fig. 6A). Bay11-7085 (16), inhibitor of IκBα phosphorylation, failed to prevent the dissociation (Fig. 6A), indicating that p53-IκBα dissociation is not due to IκBα phosphorylation and degradation. Again, Mdm2 was barely detectable in the immunoprecipitates (Fig. 6A). Staurosporine-mediated p53-IκBα dissociation, as well as p53 nuclear translocation, was also found in other types of cells such as Molt-4 T cells, breast MCF-7 cells, and prostate Du145 cells (data not shown).

To induce hypoxia, SK-N-SH cells were treated with deferoxamine for 16 h. p53-IκBα dissociation occurred at both cytosolic and nuclear levels under hypoxic conditions (Fig. 6B).

In contrast to the above effects, TGF-β1 failed to induce p53-IκBα dissociation in L929 cells when cultured in the presence of serum (Fig. 7A). TGF-β1 promotes L929 cell growth by 20–30% in 24–48 h (11). Similar results were observed using SK-N-SH cells (Fig. 7B). TGF-β1 was not growth inhibitory to SK-N-SH cells (data not shown). Nonetheless, when L929 cells had undergone serum starvation for 16–24 h and were then treated with TGF-β1 for 1 h, the expression of cytosolic IκBα was suppressed by TGF-β1 and the cytosolic p53-IκBα complex was barely detectable (Fig. 7A).

TGF-β1 mediated dissociation of the p53-IκBα complex at both cytosolic and nuclear levels in Molt-4 T cells (Fig. 7C). Similarly, TGF-β1 rapidly mediated dissociation of the cytosolic p53-IκBα complex in 1 h in freshly isolated rat spleen cells (data not shown). Lymphoid cells are sensitive to TGF-β1-mediated growth inhibition (4). TGF-β1 mediated down-regulation of IκBα in Mv1Lu cells (Fig. 2), and there was an increased nuclear p53 in these TGF-β1-treated cells (data not shown), indicating p53 undergoes nuclear translocation.

Exposure of L929 cells to etoposide, to induce DNA damage, resulted in increased p53 and IκBα accumulation in the nucleus (Fig. 8A). Etoposide mediated dissociation of the cytosolic p53-IκBα complex but increased the complex formation in the nucleus during 1-h treatment (Fig. 8A). However, at a prolonged treatment time of 16 h, continued p53 accumulation was observed in the cytoplasm and nucleus, whereas the amount of nuclear IκBα binding to p53 was reduced (Fig. 8A).

Exposure of Du145 cells to UV light, followed by culturing for 1 h, resulted in the p53-IκBα dissociation in the cytosol, as well as p53 accumulation in the nucleus (Fig. 8B). Time course studies using L929 and SK-N-SH cells also showed that UV-mediated nuclear p53-IκBα complex formation reached a plateau in 1–2 h and was then decreased (data not shown). Similar results were also observed by testing Molt-4 T cells.

The Non-ankyrin C Terminus of IκBα Physically Interacts with the Proline-rich Region and Serine 46 of p53—The Cyto trap yeast two-hybrid system was used to map the structural basis of p53 and IκBα binding interactions. The non-ankyrin C-terminal IκBα (IκBα243C) physically interacted with the full-length region, the N-terminal region (amino acids 1–100), and the proline-rich region (amino acids 66–100) of p53 (Fig. 9). However, IκBα243C could not bind the proline-rich region-deleted p53 (p53Δpro) (Fig. 9). Also, the full-length IκBα interacted with the full-length p53 but failed to bind p53Δpro (Fig. 9). These results indicate that the non-ankyrin C terminus of IκBα physi cally interacts with the proline-rich region of p53. In comparison, the N-terminal ankyrin region of IκBα could not bind the above-indicated p53 proteins (Fig. 9).

In positive controls, the binding interaction between WW-domain oxidoreductase (WOX1) and p53 (18), as well as MafB/ MafF self-interaction, was demonstrated (Fig. 9). In negative controls, no binding interactions were observed for Lamin C and p53 or empty vector and empty vector (Fig. 9).

Transcriptional activation of the mitochondrial apoptosis-inducer p53AIP1 requires serine 46 phosphorylation in p53
Deletion of serine 46 (p53/S46) abolished p53 interaction with the full-length IκBα and IκBβ (26). Alteration of serine 46 to a non-phosphorylation glycine-46 also abolished the binding interaction (Fig. 9). However, mutation of serine 46 to threonine 46, a phosphorylation site, failed to abolish the binding (Fig. 9). The observations suggest that phosphorylation of serine 46 is necessary for p53 interaction with IκBα. Also, the proline-rich region and serine 46 in p53 appear to contribute equally to the binding interaction with IκBα.

p53 and IκBα Synergistically Mediates Apoptosis—Finally, whether IκBα enhanced p53 apoptosis was examined. Transient expression of p53 and the full-length IκBα in L929 cells resulted in enhancement of p53-mediated cell death (Fig. 10A). The cells were electroporated with a non-cytotoxic dose of p53.
and IκBα. Additionally, co-expression of p53 with the full-length IκBα or IκBα243C in COS7 cells also resulted in enhancement of p53-mediated cell death (Fig. 10B). Similar results were observed by testing Mv1Lu, HeLa, and Du145 cells (data not shown).

**DISCUSSION**

Functionally, IκBα and other IκB family proteins prevent NF-κB activation by binding and sequestering NF-κB in the cytoplasm (14, 27, 28). In addition, IκBα binds the hepatitis B virus X protein and mediates nuclear import of this protein (29). Here, it is demonstrated that a portion of cytosolic IκBα interacts with p53 in vivo, as determined using various types of cells and rat liver and spleen. Notably, the cytosolic p53-IκBα complex rapidly undergoes dissociation in response to apoptotic stress, DNA damage, UV radiation, hypoxia, and TGF-β1-mediated growth suppression, indicating a regulatory control of cytosolic p53 by IκBα in resting cells. That is, IκBα plays a role in sequestering p53 in the cytoplasm and preventing p53 nuclear translocation. In response to stress stimuli, dissociation of p53-IκBα complex occurs and p53 translocates to the nucleus. Apparently, there is an IκBα-regulated p53 pool in resting cells and this p53 readily translocates to the nucleus in response to exogenous stress.

Unlike Mv1Lu and lymphoid cells, TGF-β1 could not inhibit the growth of L929 fibroblasts and neuronal SK-N-SH cells. TGF-β1 failed to mediate the dissociation of the cytosolic p53-IκBα complex. Indeed, the amount of cytosolic p53-IκBα complex was increased by TGF-β1 in L929 cells. Presumably, during growth response, a strict control of cytosolic p53 by IκBα is needed for preventing p53 nuclear translocation and its induction of p21 for growth suppression (25).

As a tumor suppressor protein, p53 controls cell growth, repairs damaged DNA, maintains mitochondrial biogenesis, and mediates apoptosis (25). In unstimulated resting cells, the cytosolic and nuclear p53 levels are very low. A proposed model for p53 turnover in resting cells is that a portion of cytosolic p53 complexes with IκBα and the complex may constantly dissociate to allow p53 nuclear translocation for maintaining optimal cellular function. The nuclear p53 then complexes with Mdm2 and is exported to the cytoplasm for ubiquitination and degradation (25). Newly synthesized p53 again complexes with IκBα for serving as a new cytosolic stock.

An increased p53-IκBα complex formation was observed in the nucleus during exposure of cells to etoposide and UV for 1 h. However, this event did not occur when cells were treated with staurosporine, indicating that specific exogenous signals are needed for the nuclear p53-IκBα complex formation. When cells were treated for a prolonged time with etoposide, dissociation of the nuclear p53-IκBα complex occurred, along with simultaneous p53 accumulation in the nucleus. The event is apparently necessary for p53-mediated apoptosis.

p53 and IκBα synergistically enhance cell death during transient co-expression. To elucidate the underlying mechanism, it is necessary to determine the kinetics of complex formation of ectopic IκBα and p53 in both cytoplasm and nucleus before and at the time of cell death. Also, inhibition of NF-κB, an anti-apoptotic factor (12), by ectopic IκBα is likely. p53 apoptotic function may be enhanced due to suppression of NF-κB function by IκBα. Nonetheless, the notion is not supported by a recent study showing that NF-κB is a co-factor of p53 in mediating cell death (30).

Dissociation of the cytosolic p53-IκBα complex is not due to IκBα phosphorylation and subsequent ubiquitination for degradation. Inhibition of IκBα phosphorylation by Bay11-7085 could not prevent staurosporine-mediated p53-IκBα dissociation. Additionally, exposure of cells to EDTA failed to dissociate the cytosolic p53-IκBα complex, indicating that the p53-IκBα interaction is calcium-independent. Nonetheless, the experiments from the yeast two-hybrid interaction system showed that phosphorylation of serine 46 in p53 may be involved in the p53-IκBα complex formation. Dissociation of this complex may be due to de-phosphorylation of serine 46 in p53, when cells are exposed to exogenous stress stimuli.

Structurally, IκBα has a surface-exposed N terminus, a central, protease-resistant domain containing five ankyrin repeats, and a compact, highly acidic PEST domain-containing C terminus (27). The ankyrin repeats are involved in the binding of IκBα with NF-κB/Rel proteins. IκBα nuclear localization is independent of NF-κB/Rel proteins, and the second ankyrin repeat of IκBα has been shown to be responsible for nuclear localization (28). Additionally, importins α and β, the small GTPase Ran, and unidentified proteins that interact with the ankyrin repeats are involved in nuclear transport of IκBα (27). Nuclear IκBα inhibits the interaction of NF-κB with target DNA and promotes the export of NF-κB from the nucleus to the cytoplasm (27). The first ankyrin repeat of IκBα has the strongest inhibitory effect on NF-κB activation (14).

Two pathways are involved in IκBα degradation. One pathway is the TNF-mediated activation of IKK kinases, which phosphorylates IκBα (31). The phosphorylated IκBα dissociates from the NF-κB-IκBα complex and is degraded by the protea-
some/ubiquitin pathway. A parallel pathway is the TNF activation of cytosolic calpains, which degrades IκB and activates NF-κB independently of the ubiquitin/proteasome pathway (32, 33). In contrast to these observations, constitutive nuclear translocation of NF-κB in B cells fails to result in degradation of IκB proteins (34). Unlike inflammatory cytokines, hypoxia, reoxygenation, and the tyrosine phosphatase inhibitor pervanadate activate NF-κB and tyrosine phosphorylation of IκBα, whereas this event could not induce degradation of IκBα by the proteasome/ubiquitin pathway (34–36).

Whether TGF-β1-mediated down-regulation of cytosolic IκBα in Mv1Lu cells is due to degradation by the proteasome/ubiquitin pathway or due to inhibition of gene transcription by the Smad protein complex remains to be determined. Previously we have shown that TIAF1, TGF-β-induced antip apoptotic factor, inhibits IκBα expression when overexpressed in L929 cells (9). TIAF1 suppresses gene expression of IκBα.2 TGF-β1 is not an activator of NF-κB, and both proteins may counteract each other’s functions (6, 12, 37–39). Thus, inhibition of IκBα expression by TGF-β1 in Mv1Lu cells is likely due to TIAF1-suppressed gene expression rather than IκBα degradation by the proteasome/ubiquitin system. Suppression of IκBα expression by TGF-β1 allows p53 nuclear translocation for exerting growth arrest and apoptosis.

The functional significance of IκBα interaction with the proline-rich or growth regulatory region of p53 is unknown. Berger et al. (20) showed that p53 without the proline-rich region becomes more susceptible to ubiquitination, nuclear export, and Mdm2-mediated degradation, indicating that this region is necessary for p53 protein stability. We demonstrated that WOX1 interacts with p53 at the proline-rich or growth regulatory region (18). Most recently, we found that WOX1 may allow p53 nuclear translocation for exerting growth arrest and apoptosis.

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