**IκB Kinase α Regulates Subcellular Distribution and Turnover of Cyclin D1 by Phosphorylation**

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IKK kinases (IKKs), IκKα and IκKβ, with a regulatory subunit IκKγ/NEMO constitute a high molecular weight IKK complex that regulates NF-κB activity. Although IκKα and IκKβ share structural and biochemical similarities, IκKα has been shown to have distinct biological roles. Here we show that IκKα plays a critical role in regulating cyclin D1 during the cell cycle. Analysis of IκKα−/− mouse embryo fibroblast cells showed that cyclin D1 is overexpressed and localized in the nucleus compared with parental mouse embryo fibroblast cells. IκKα associates with and phosphorylates cyclin D1. Analysis on cyclin D1 mutants demonstrated that IκKα phosphorylates cyclin D1 at Thr286. Reconstitution of IκKα in knockout cells leads to nuclear export and increased degradation of cyclin D1. Further, RNAi-mediated knockdown of IκKα results in similar changes as observed in IκKα−/− cells. These results suggest a novel role of IκKα in regulating subcellular localization and proteolysis of cyclin D1 by phosphorylation of cyclin D1 at Thr286, the same residue earlier found to be a target for glycosyn synthase-3β-induced phosphorylation.

The IκKγ complex is the major regulatory component in the NF-κB pathway that is activated by a variety of stimuli, including tumor necrosis factor α, interleukin-1, lipopolysaccharide, UV radiation, and stress (1–4). NF-κB transcription factors are sequestered in the cytoplasm in association with the inhibitory protein called IκB. The IKK complex phosphorylates IκB, which leads to its ubiquitination, followed by proteosomal degradation. The released NF-κB transcription factors are transported to the nucleus and activate or repress expression of genes involved in a variety of biological activity including inflammation, immune response, cell growth, and apoptosis. The IκK complex includes the catalytic subunits IκKα and IκKβ, and a regulatory subunit IκKγ/NEMO (5–10). Despite their structural and biochemical similarities, both IκKα and IκKβ are functionally distinct (11, 12). IκKβ is the major kinase for NF-κB activation in canonical pathway, and the role of IκKα is considered redundant in this process. Analysis of IκKα knock-out mice reveals a distinctive phenotype, including severe defects of skin and limb development, and IκKα has been shown to be involved in the development of the epidermis (13–15), a role possibly independent of NF-κB (16, 17). Mechanisms responsible for these phenotypic changes observed in knockout mice remain poorly understood. IκKα activity is required for both B-cell maturation (18, 19) and mammary gland development (20), which are dependent on processing of the NF-κB2/p100 precursor to the mature p52 subunit and cyclin D1 transcription in response to RANK ligand in mammary epithelial cells, respectively. Recently, IκKα was shown to regulate cytokine-inducible gene expression through its association with NF-κB-regulated promoters and histone H3 phosphorylation (21–23).

D-type cyclins (cyclin D1, D2, and D3) are critical for entry of cells into cell cycle and mediate G1 to S phase progression (24–28). Cyclin D with its partner cyclin-dependent kinases (CDKs), CDK4 and CDK6, phosphorylate their downstream target, the retinoblastoma tumor suppressor protein (pRb). pRb phosphorylation releases the E2F family of transcription factors that activate the expression of cyclin E and multiple other genes required for the cell cycle progression (3, 29, 30). Association of cyclin E with CDK2 leads to further phosphorylation of pRb, thus providing a positive feedback loop for progression of the cell cycle beyond the G1/S checkpoint. Different mitogenic pathways, including Ras/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase, induce expression of cyclin D, whereas negative regulators of the cell cycle cause phosphorylation of cyclin D by glycosyn synthase kinase (GSK)-3β with subsequent ubiquitination and degradation (31–34).

IκKα has been shown to induce cyclin D expression in mammary epithelium by RANK/RANKL-mediated NF-κB activation (20). Disordered epidermal cell proliferation in IκKα−/− mice also suggested a role of IκKα in cellular proliferation (14, 15). These observations led us to investigate the role of IκKα in cell cycle regulation. Subcellular distribution of cyclin D1 is cell cycle-specific. Newly synthesized cyclin D1 in association with CDK4/CDK6 is transported to the nucleus to induce pRb phosphorylation. During the S phase, cyclin D1 becomes exclusively cytoplasmic. Nuclear export of cyclin D1, subsequent ubiquitination, and proteolysis are dependent on GSK-3β-induced phosphorylation of cyclin D1 at the Thr286 residue. We found that in IκKα−/− cells, cyclin D1 was localized in the nucleus and was more stable than that in parental MEF cells. Reconstitution of IκKα−/− cells with IκKα restores these defects found in IκKα−/− cells. Further, we demonstrate that IκKα associates with cyclin D1 and phosphorylates it at Thr286. Thus in addition to the earlier demonstrated role of IκKα in transcriptional regulation of cyclin D1, our data indicate that IκKα regulates cyclin D1 at the post-transcriptional level as well. It induces phosphorylation of cyclin D1, leading to the nuclear export and subsequent proteolysis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Wild-type MEF cells were a kind gift from Xiaodong Wong. IκKα−/− and IκKβ−/− cells were kindly provided by Inder M. Verma (15, 35). A Moloney-based retrovirus expressing Myc-tagged IκKα or β-galactosidase was utilized with hygromycin selection to generate stable cell lines from parental IκKα knockout cells, expressing...
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either IKKα (IKKα+/+) or β-galactosidase (IKKα−/−), and has been described previously (23). IKKα−/− cells thus generated were similar to parental IKKα knockout cells and were used to perform different experiments. HeLa and 293 cells were obtained from ATCC. All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum.

**Plasmids**—Epitope-tagged IKKα and IKKB wild-type and point mutants were previously described (7, 12). Charles Sherr kindly provided bacterial expression vectors encoding cyclin D1 or CDK4. The full-length cyclin D1 cDNA was cloned into pGEX-5X-1 at the BamHI-EcoRI sites. Truncated cyclin D1 fragment spanning residues 1–267 was subcloned by self-ligation following digestion of full-length cyclin D1 in pGEX-5X-1 with Sall restriction enzyme. The C terminus NcoI-XhoI fragment spanning residues 201–295 was subcloned into pGEX-KG vector. To make a construct encoding amino acids 201–262, a nonsense mutation substituting an amber stop codon (TAG) for a Gln codon (CAG) at positions 263 and 264 in cDNA encoding aa 201–295 of cyclin D1 was introduced by PCR mutagenesis using a site-directed mutagenesis kit (Stratagene). Threonine residues in C terminus (aa 201–295) or full-length cyclin D1 were substituted with alanine, either individually or in combination. CMV expression vector encoding CDK4 was cloned into either FLAG-tagged or Myc-tagged pCMV5 at the BglII and SmaI sites. Cyclin D1 cDNA was cloned into both FLAG-tagged and Myc-tagged pCMV5 at the BamHI-Smal and KpnI-BamHI sites, respectively. Full-length Bb cDNA was cloned into the BamHI and Xhol sites of pGEX-4T-3. All cDNA constructs were confirmed by DNA sequencing. Different GST fusion proteins were expressed in *Escherichia coli* and purified as described earlier (36).

**Immunoprecipitation, Immunoblotting, and Kinase and GST Pull-down Assays**—These assays were performed as described earlier (36, 37). The following antibodies were used during these assays: IKKα (OP-133; Oncogene), IKKB (sc-7607; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), cyclin D1 (sc-718 and sc-72–13G; Santa Cruz Biotechnology), cyclin E1 (sc-481; Santa Cruz Biotechnology), CDK6 (sc-177; Santa Cruz Biotechnology), CDK7 (sc-529; Santa Cruz Biotechnology), cyclin E1 (sc-481; Santa Cruz Biotechnology), and actin (sc-1615; Santa Cruz Biotechnology).

**Quantitative Real Time PCR**—Real-time PCR was performed for expression of cyclin D1 mRNA using total RNA prepared from different MEF cells, following a procedure as described earlier (36). In brief, cDNA was prepared with oligo(dT) and random primers (Invitrogen) from total RNA extracted from these cells at different time points following serum stimulation. For real-time PCR, the prepared cDNA was analyzed in triplicate in a 20-μl volume with the SYBR Green Master Mix (Applied Biosystems) for 40 cycles in the ABI sequence detection system. The oligonucleotide primers used to analyze transcripts were as follows: cyclin D1, aa 675–692 (5′-CTG CTA CGG CAC ACG GCA-3′) and aa 725–741 (5′-GCA GCC ACG GAC GTA-3′); gcleraldehyde-3-phosphate dehydrogenase, aa 823–843 (5′-TGA AGG CAT CTG AGG G-3′) and aa 879–900 (5′-TGG AAG TCG CAG GAC ACC-3′). Cyclin D1 mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA.

**Chromatin Immunoprecipitation (ChIP) Assays**—ChIP assays were performed following a previously described protocol (23, 38) based on the manufacturer’s suggested protocol (Upstate Biotechnology, Inc., Lake Placid, NY). Briefly, chromatin from cross-linked cells that was sheared by a sonicator (3 × 15 s; one-third power) was incubated with specific antibody for overnight followed by incubation with salmon sperm DNA-saturated protein G-Sepharose. Precipitated DNAs were analyzed by quantitative PCR (34 cycles) using a TaqPCR Master mix kit (Qiagen) and oligonucleotide primers for either the murine cyclin D1 promoter 5′−CCT CAC TGC TCC CGA GCC-3′ (−151 to −134) and 5′−TGG AGG CTG CAG GAC TTT GC-3′ (−1 to +19) or murine IkBa promoter site 5′−GGA CCC CAA ACC AAA ATC G-3′ (−316 to −308) and 5′−TCA GGC GGG GAT TTC C-3′ (−15 to +4).

**In Vivo Cell Labeling**—For in vivo phosphate labeling of cyclin D1, MEF cells were incubated with serum-free Dulbecco’s modified Eagle’s medium without sodium phosphate for 6 h. Then cells were labeled with [32P]orthophosphoric acid (PerkinElmer Life Sciences) at a final concentration of 50 μCi/ml at 37 °C for 3 h. Labeling of the cyclin D1 was determined following immunoprecipitation of whole cell lysates with cyclin D1 antibody and autoradiography.

**Immunofluorescence Microscopy**—Immunofluorescence microscopy was performed on different MEF cells and HeLa cells after RNA interference following a method described earlier (23). In different MEF cells, cyclin D1 staining was performed on cells synchronized by serum starvation for 36 h followed by culture in serum-containing medium for variable time points up to 24 h. HeLa cells were used to determine cyclin D1 localization 72 h after transfection with siRNA to IKKα, IKKB, and tax used as a control. BrdUrd staining was performed on cells labeled with BrdUrd for 16 h.

**RNA Interference**—RNA interference-induced inhibition of IKKα and IKKB was performed by transfection of siRNA cognate to IKKα (5′-CUU CAG GAA CAU CAC AGG CtdTdT-3′), IKKB (5′-GGU GGA AGA GGU GGU GAG CtdTdT-3′), and HTLV-1 tax (5′-GAU GGA GCC GUU AUC GCC UdTdT-3′) as a control (Dharmacon Research Inc.) following a method as described earlier (39). In brief, HeLa cells at 30–40% confluence were transfected with annealed siRNA duplexes using Oligofectamine (Invitrogen) at a final RNA concentration of 20 nm. 48 h after transfection of siRNA, cells were used for immunofluorescence microscopy, or whole cell lysate was prepared for immunoblotting.

**RESULTS**

**Cyclin D1 Is Constitutively Localized in the Nucleus in IKKα−/− Cells**—Cyclin D1 localization in nuclear-cytoplasmic compartments varies with cell cycle and becomes exclusively cytoplasmic during the S phase (32, 40). Earlier studies have shown that subcellular localization of cyclin D1 is regulated by GSK-3β (24, 32, 33). We and other groups recently have shown that IKKα shuttles between cytoplasm and nucleus (22, 23). Therefore, we investigated whether IKKα affects subcellular distribution of cyclin D1. Parental MEF, IKKα−/−, IKKB−/−, and IKKα+/+ cells were synchronized by serum starvation for 48 h and stimulated to undergo active cycling by the addition of serum for 24 h. Cells were harvested at 0 and 24 h after serum stimulation with serum (Fig. 1A). In contrast, it was exclusively localized in the nucleus of IKKα−/− cells in both serum-starved cells as well as after stimulation with serum (Fig. 1A). To further confirm that nuclear localization of cyclin D1 in IKKα−/− cells is due to the absence of IKKα, we evaluated cyclin D1 localization in IKKα reconstituted (IKKα+/+) cells. The expression of IKKα in these cells was confirmed previously (23) and in this study by immunoblotting as seen in Fig. 2A. Most of the IKKα+/+ cells showed cyclin D1 in cytoplasmic distribution that is similar to that of MEF cells. These results indicate that IKKα is critical for nuclear export of cyclin D1 during the cell cycle.

Cyclin D1 can be predominantly localized in the nucleus of quiescent cells. Early cell cycle exit of IKKα−/− cells may result in observed nuclear localization pattern of cyclin D1 in these cells. To exclude this possibility, we performed BrdUrd labeling of asynchronously prolifer-
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FIGURE 1. IKKα regulates transport and expression of cyclin D1. A, immunofluorescence photomicrographs of MEF cells stimulated with serum for 24 h following serum starvation for 48 h, labeled with cyclin D1 antibody and rhodamine (red)-conjugated secondary antibody. B, unsynchronized MEF cells were grown in the presence of BrdUrd for 24 h, and the cells were stained with both cyclin D1 and BrdUrd antibody. Cyclin D1 and BrdUrd were labeled with rhodamine (red)- and fluorescein isothiocyanate (green)-conjugated secondary antibody, respectively.

FIGURE 2. IKKα regulates cyclin D1 expression. A, cells were stimulated with serum for 6 h after starvation for 48 h. Cell lysates were prepared and then subjected to Western blot (WB) analysis with the indicated antibodies. The same cell lysates were used to determine the expression level of cyclin D1 mRNA by reverse transcription followed by PCR (B) or real time PCR (C). Cyclin D1 mRNA levels were normalized with glyceraldehyde-3-phosphate dehydrogenase mRNA in real time PCR analysis. Bars, S.D. D, cells were treated with serum for 6 h, and chromatin cell lysates were prepared. ChIP assays were performed by immunoprecipitation with p65 antibody or IgG and then PCR with murine cyclin D1 promoter-specific primers. As a control, cells were treated with tumor necrosis factor α (TNFα) for 30 min and subjected to p65 immunoprecipitation followed by PCR with murine lkb1 promoter-specific primers. Input, 10% of DNA used for ChIP assays.

Cyclin D1 was mainly localized in the nucleus of IKKα−/− cells, even those incorporating BrdUrd as compared with other cells that showed cyclin D1 distribution either in the cytoplasm or the nucleus (Fig. 1B). These results demonstrate that IKKα−/− cells enter into the S phase as demonstrated by BrdUrd incorporation, and even during the S phase, cyclin D1 remains nuclear in these cells. These results suggest that cyclin D1 nuclear localization in IKKα−/− cells is directly related to lack of IKKα.

IKKα−/− Cells Show Overexpression of Cyclin D1—Current studies suggest that cell cycle phase-specific expression of different cyclins drives the cell cycle (28). The levels of G1 cyclins such as cyclin D or cyclin E are increased during the G1/S phase of the cell cycle (25, 28). We determined whether impaired subcellular localization of cyclin D1 is associated with the change in the level of cyclin D1 expression. The indicated cells were starved for 48 h and subjected to Western blotting to determine expression of cyclin D1 and various other proteins known to play a role during early phases of cell cycle progression. Results showed that contrary to the expectation, cyclin D1 levels were markedly increased in IKKα−/− cells (5.6-fold higher) as compared with that of MEF or IKKβ−/− cells both at 0 and 6 h following serum stimulation (Fig. 2A). Cyclin D1 overexpression in IKKα−/− cells remained up to 24 h of culture in the presence of serum without significant change (data not shown). However, levels of E2F-regulated proteins, cyclin E and E2F, were lower in IKKα−/− cells as compared with MEF or IKKβ−/− cells. Other G1/S-specific proteins, including cyclin D partner kinases (CDK4 and CDK6) were similar in all cell lines. Increased accumulation of cyclin D1 in IKKα−/− cells was significantly decreased (50% of IKKα−/−) in IKKα−/− cells (Fig. 2A).

We addressed whether the observed accumulation of cyclin D1 in IKKα−/− cells is mediated by transcriptional regulation via IKKα. Total RNA was prepared from the cells that had been stimulated with serum for the indicated time following starvation as above. Reverse transcription followed by PCR was performed to determine the expression of cyclin D1 mRNA. The level of cyclin D1 mRNA was increased following serum stimulation in MEF cells as previously reported in other cell lines (20, 41). However, the cyclin D1 mRNA levels in IKKα−/− cells were much higher than that of MEF or IKKβ−/− cells (Fig. 2B). The increased cyclin D1 mRNA levels in IKKα−/− cells were markedly reduced in IKKα-reconstituted IKKα−/− cells. Similar results were seen with quantitative real time PCR analysis (Fig. 2C), indicating that cyclin D1 mRNA is up-regulated in IKKα−/− cells.
These results indicate that IKKᵦ was a marked increase in recruitment of p65 to the IκBα promoter, and this association was more prominent in IKKᵦ cells than in control cells. These results suggest that cyclin D1 is a novel substrate for IKKᵦ kinase activity in vivo. Therefore, we hypothesized that IKKᵦ regulates nuclear export of cyclin D1 by phosphorylation. In vivo, IKKᵦ and IKKβ interfere with cyclin D1 nuclear export by phosphorylation at specific residues on cyclin D1. Hence, the reduced cyclin D1 phosphorylation in IKKᵦ⁻/⁻ cells was restored in IKKᵦ⁻/⁻ cells (Fig. 4C). Given that cyclin D1 is a potent inhibitor of NF-κB activation, the reduced phosphorylated cyclin D1 levels as compared with that of MEF, IKKα⁻/⁻, or IKKβ⁻/⁻ cells indicates a much lower ratio of phosphorylated versus unphosphorylated cyclin D1 levels in IKKα⁻/⁻ cells (Fig. 4C). The results indicate that IKKα mediates cyclin D1 phosphorylation both in vivo and in vitro.\n\nIKKα interacts with and Phosphorylates Cyclin D1 in vivo. Previous reports have shown that NF-κB activates cyclin D1 gene expression. Thus, we determined whether NF-κB regulates increased expression of cyclin D1 in IKKα⁻/⁻ cells using ChIP assays. Chromatin lysates were prepared from serum-treated cells and subjected to immunoprecipitation with p65 antibody followed by PCR with cyclin D1-specific primer. ChIP analysis indicated that p65 was recruited to cyclin D1 promoter and this association was more prominent in IKKα⁻/⁻ cells. No significant change was observed after exposure to serum. Recruitment of p65 to IκBα promoter was used as a control. As expected, there was a marked increase in recruitment of p65 to the IκBα promoter in parental MEF and IKKα-reconstituted cells; however, there was no significant change in IKKα⁻/⁻ and IKKβ⁻/⁻ cells. These results suggest that increased cyclin D1 expression in IKKα⁻/⁻ cells is secondary to increased p65 association to cyclin D1 promoter in the absence of IKKα.

IKKα Interacts with and Phosphorylates Cyclin D1 in IKKα⁻/⁻ cells. We next determined the ability of IKKα to phosphorylate cyclin D1 protein. In vitro kinase assay was performed with baculovirus-expressed IKKα using GST-cyclin D1 as a substrate. Affinity-purified IKKα was able to phosphorylate cyclin D1 (Fig. 4A, left), as was GSK-3β, used as a positive control (Fig. 4A, middle). Kinase-inactive mutants of IKKα did not phosphorylate cyclin D1, indicating that IKKα kinase activity is required for cyclin D1 phosphorylation (Fig. 4B). Next, we determined whether cyclin D1 could be phosphorylated by IKKα in vivo. MEF, IKKα⁻/⁻, IKKβ⁻/⁻, and IKKα⁻/⁻ cells were labeled with [32P]orthophosphoric acid for 3 h following incubation with phosphate-deficient medium for 6 h. Cell lysates were prepared and then subjected to immunoprecipitation with cyclin D1 antibody followed by autoradiography. The results showed that endogenous cyclin D1 in MEF cells was significantly phosphorylated, whereas it was markedly reduced in IKKα⁻/⁻ cells. The reduced cyclin D1 phosphorylation in IKKα⁻/⁻ cells was restored in IKKα⁻/⁻ cells (Fig. 4C). Given that cyclin D1 is a potent inhibitor of NF-κB activation, the reduced phosphorylated cyclin D1 levels as compared with that of MEF, IKKα⁻/⁻, or IKKβ⁻/⁻ cells indicates a much lower ratio of phosphorylated versus unphosphorylated cyclin D1 levels in IKKα⁻/⁻ cells (Fig. 4C). The results indicate that IKKα mediates cyclin D1 phosphorylation both in vivo and in vitro.

IKKα Phosphorylates Carboxyl Terminus of Cyclin D1 at Threonine 286—Cyclin D1 contains multiple domains responsible for its biological function (Fig. 5A). Specific residues in cyclin D1 that are phosphorylated have been identified previously. These include the threonine 286 residue by Mirk, and phosphorylation at these sites regulates the cyclin D1 amount and subcellular distribution during the cell cycle (32, 42–44). We determined specific residues in cyclin D1 that are targets for phosphorylation by IKKα using the in vitro kinase assay. The analysis of various cyclin D1 mutants with deleted amino and carboxyl terminus demonstrated that IKKα phosphorylates cyclin D1 wild type and carboxyl terminus mutant (aa 201–295) that contains the PEST domain (aa 263–295) (Fig. 5B). Potential sites for IKKα phosphorylation in cyclin D1 mutant (aa 201–295) include threonine residues 271, 286, and 288 within the PEST domain. Each of these threonine residues were mutated to alanine in both full-length and carboxyl terminus GST-cyclin D1 constructs. Cyclin D1 phosphorylation by IKKα was significantly reduced in the truncated
proteins in which the threonine residue at 286 was substituted with alanine (Fig. 5C). A similar pattern of phosphorylation by IKKα was observed when full-length GST-cyclin D1 mutants were used as substrates (Fig. 5D). In vivo labeling of MEF cells transfected with wild-type or mutant cyclin D1 showed that phosphorylation of cyclin D1 T286A mutant was significantly reduced compared with wild-type protein (Fig. 5E). These results indicate that IKKα phosphorylates the threonine 286 residue in the PEST domain of cyclin D1 and shares the same substrate specificity as described for GSK-3β (31).

Cyclin D1 is required for activity of its cognate cyclin-dependent kinases CDK4 and CDK6. Phosphorylation of cyclin D1 might be important for CDK4 kinase activity. Therefore, we evaluated kinase activity of CDK4 from MEF and IKKα−/− cells. Cell lysates were prepared and immunoprecipitated with CDK4 antibody, and then subjected to in vitro kinase assay using GST-Rb as a substrate. CDK4 immunoprecipitated from MEF cells was able to phosphorylate Rb, whereas that from IKKα−/− cells revealed decreased Rb phosphorylation (Fig. 5F). Reconstitution of IKKα knockout cells (IKKα+/−) with IKKα completely restored the CDK4-mediated Rb phosphorylation (Fig. 5F, left). Unrelated kinase CDK7 activity was not significantly different in these cells when the carboxyl-terminal domain (CTD) of RNA polymerase II was used as substrate (Fig. 5F, right). These results indicate that IKKα regulates cyclin D1-CDK4 kinase activity on pRb, possibly by post-translational modifications of cyclin D1 via phosphorylation.

IKKα Regulates the Stability of Cyclin D1—Previous studies demonstrated that GSK-3β phosphorylates cyclin D1 at threonine 286 and marks it for ubiquitination and proteasomal degradation (31, 32). Since IKKα phosphorylated the same threonine residue in cyclin D1 protein, it was important to investigate whether IKKα affects the turnover of cyclin D1. To determine the turnover of cyclin D1 in MEF, IKKα−/−, IKKβ−/−, and IKKα+/− cells, these cells were treated with cycloheximide for the indicated times. Afterward, the cells were harvested, and lysates were prepared for Western blotting with cyclin D1 antibody. This analysis revealed that there is a considerably increased half-life of cyclin D1 in IKKα−/− cells as compared with that in MEF or IKKβ−/− cells (Fig. 6A). IKKα-reconstituted cells exhibited a reduced half-life of cyclin D1 similar to that in MEF cells. The cyclin D1 amount at indicated time points after cycloheximide treatment, as measured by densitometric scanning of immunoblots, is plotted in Fig. 6B. Increased cyclin D1 half-life is specifically caused by IKKα, since IKKβ−/− cells had a cyclin D1 half-life similar to that in counterpart MEF cells. These results indicate that IKKα, but not IKKβ, regulates and is required for cyclin D1 proteolysis.

RNA Interference-mediated Knockdown of IKKα Mimics Changes Observed in IKKα−/− Cells—To further substantiate the finding that nuclear localization and overexpression of cyclin D1 in IKKα−/− cells was mediated by IKKα, siRNA-induced inhibition of IKKα was utilized. siRNA specific to the HTLV-1 tax gene was used as a control. HeLa cells were transfected with IKKα-, IKKβ-, or tax-specific siRNA oligonucleotides, and the cells were harvested at 72 h post-transfection. Cell lysates were prepared and subjected to immunoblotting. The results indicated that the IKKα and IKKβ levels in these extracts were specifically reduced in the presence of IKKα and IKKβ siRNAs, respectively, whereas the levels of IKKα and IKKβ were not affected by transfection of tax siRNA (Fig. 7A). These siRNAs had no significant effect on the expression levels of other endogenous genes, α-actin, or CDK4 (Fig. 7A). These results indicate the specificity of siRNA in decreasing either IKKα or IKKβ expression. Immunostaining with cyclin D1 antibody revealed that cyclin D1 levels were increased in the presence of IKKα-specific siRNA. In contrast, there was no significant decrease in cyclin
cyclin D1 is a substrate for IKK and chromatin remodeling (22, 23). In this study, we have identified that injected to transfection with siRNA to IKK treatment resulted in nuclear distribution of cyclin D1 in most of the cells. Recently, it has been noted that IKK siRNAs (Fig. 7 A) phosphorylation was reduced by IKK treatment. Western blot (WB) shows that IKK mediates protein degradation of cyclin D1. Our results indicate that IKK regulates subcellular localization and turnover of cyclin D1 by phosphorylation of cyclin D1 at Thr286.

DISCUSSION

IKKα was identified as one of the catalytic subunits of IKK complex that mediates the NF-κB activation. In addition to IKKα, IKKβ and a regulatory subunit IKKγ/Nemo constitute the IKK complex. IKKβ appears to be the predominant kinase mediating IkB phosphorylation in response to most of the upstream signals leading to NF-κB activation. Recently, it has been noted that IKKα has distinct biological functions unrelated to IkB phosphorylation. These include p100 processing (18) and chromatin remodeling (22, 23). In this study, we have identified that cyclin D1 is a substrate for IKKα. Our results demonstrated that IKKα can interact with and phosphorylate cyclin D1 at the threonine 286 residue within the PEST domain. IKKα−/− cells showed exclusive nuclear localization and increased stability of cyclin D1 protein compared with parental MEF cells. Reconstitution of IKKα−/− cells with IKKα resulted in reduced expression and cytoplasmic distribution of cyclin D1, a pattern similar to that observed in MEF cells. siRNA-mediated knockdown of IKKα but not IKKβ mimics the cyclin D1 pattern to that observed in IKKα−/− cells. These results demonstrate that IKKα regulates subcellular localization and turnover of cyclin D1 by phosphorylation of cyclin D1 at Thr286.

It was previously reported that GSK-3β regulates subcellular localization, ubiquitination, and proteolysis of cyclin D1 (32, 44, 45). Cyclin D1 mutant that substitutes threonine 286 with alanine (T286A) is constitutively localized in the nucleus with impaired degradation via the ubiquitin-proteosome pathway. Similarly, a splice variant of cyclin D (cyclin D1b), which lacks the carboxyl-terminal domain, including threonine 286, was distributed in the nucleus (42) and demonstrates increased stability as well (42, 43). These results suggest that phosphorylation at the threonine 286 residue is critical for nuclear export and degradation of cyclin D1. Our results indicate that IKKα phosphorylates the same threonine residue in cyclin D1 earlier reported to be a target for GSK-3β-induced phosphorylation. Earlier data suggest that GSK-3β is the physiological kinase mediating cyclin D1 phosphorylation (32).

Several possibilities could explain the role of IKKα and GSK-3β in inducing cyclin D1 phosphorylation. It is possible that both GSK-3β and IKKα mediate cyclin D1 phosphorylation in a context-dependent manner similar to β-catenin (41, 46) that is a substrate for both GSK-3β and IKKα. Other possibilities include (a) an interplay between these two pathways to mediate cyclin D phosphorylation or (b) cyclin D1 being subject to sequential or progressive phosphorylation by more than one kinase. We used baculovirus-expressed affinity-purified IKKα in in vitro kinase assays, thus reducing possibility of GSK-3β contamination. Additionally, nuclear localization of cyclin D1 in IKKα−/− cells, a process dependent on cyclin D1 phosphorylation, strongly suggests that
IKKα directly phosphorylates cyclin D1 but does not conclusively prove that this is the case in vivo under physiological conditions. GSK-3β knockout mice suffer embryonic lethality due to an inability to activate NF-κB, a phenotype similar to that observed in IKKβ−/− and p65−/− mice (47). In another study, it was observed that activation of NF-κB is impaired in response to multiple stimuli in GSK-3β−/− MEF cells with the possibility of GSK-3β acting upstream of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase (48). So far, studies point toward GSK-3β being upstream of IKKα. It remains to be seen whether GSK-3β could be a downstream target of IKKα either directly or through intermediate kinases. Of note, in the study mentioned above by Takada et al. (48), there was no increased accumulation of cyclin D1 in GSK-3β−/− cells, suggesting the possibility of an alternate kinase mediating cyclin D1 phosphorylation and degradation.

Transcriptional up-regulation of cyclins coupled with inhibition of proteolysis leads to cyclin accumulation that drives cells through specific phases of the cell cycle (24, 25, 28). Once cells pass through the requirement of specific cyclins, they are rapidly degraded. GSK-3β activity is increased under adverse cell proliferative conditions such as starvation, thus maintaining cells in quiescent phase, whereas under mitogenic conditions, the activity of GSK-3β is inhibited by the Ras signaling pathway involving PI-3K and AKT (32), thereby allowing cyclin D1 to be stable and resulting in progression of the cell cycle. On the other hand, IKKα may be required for cyclin D1 phosphorylation during the S phase in actively cycling cells. The possibility of cross-talk between these pathways during the cell cycle may exist. In addition to GSK-3β, it has been reported that Mirk/dyrk1B, an arginine-directed serine/threonine kinase, phosphorylates cyclin D1 at the threonine 288 residue, which results in cyclin D1 destabilization in G0/G1 (49). It appears that Mirk in conjunction with GSK-3β leads to cell cycle arrest by cyclin D degradation.

Prior studies with cyclin D1b and cyclin D1-T286A mutant indicated that the carboxyl-terminal domain is important for cellular transformation and tumorigenesis (33, 42–44). Cells containing cyclin D1b proliferate slowly but are tumorigenic, although cyclin D-CDK4 immunoprecipitated from these cells demonstrated poor Rb phosphorylation (42, 43). It has been suggested that the oncogenic potential of cyclin D1b and the cyclin D1-T286A mutant could be attributed to their constitutive localization. These findings are similar to our preliminary observations in that IKKα−/− cells proliferate slowly (3) and cyclin D-CDK4 from these cells is inefficient in phosphorylating Rb (Fig. 4F). BrdUrd labeling of IKKα−/− cells and cell cycle kinetics suggests that IKKα−/− cells demonstrate delayed transition to S phase despite abundant cyclin D1. This raises a possibility that cyclin D1 present in IKKα−/− cells has impaired activity, consistent with reduced phosphorylation of GST-Rb by CDK4-cyclin D1 from IKKα knockout cells. It is also possible that IKKα-induced cyclin D1 phosphorylation may be required for either its association with partner CDK or kinase activity of the complex. However, other possible mechanisms could explain slower proliferation of IKKα−/− cells and cells with a cyclin D1 splice variant. Unlike other cyclins, a significant amount of cyclin D in the resting cells (50–52) also suggests that a post-translational event may be required to make cyclin D1 active and initiate G1/S progression. Earlier studies with GSK-3β do not suggest that GSK-3β-induced phosphorylation of cyclin D1 affects its activity. It has been described earlier that assembly of newly synthesized cyclin D1 with CDK4 depends on a kinase cascade that is activated by Raf-1, mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2, and extracellular signal-regulated kinase (32, 53, 54). Although not addressed in our current work, it raises the possibility that there might be cross-talk between Raf signaling pathway and IKKα. It would be critical to find out whether IKKα affects cyclin D activity and mechanisms involved such as those by phosphorylation at additional sites, recruitment of other proteins, or activity on other cell cycle regulatory proteins. Further work defining upstream pathways that lead to IKKα-induced cyclin D phosphorylation should provide critical insight into this novel mechanism of G1/S regulation.

Up-regulation of NF-κB through multiple stimuli leads to overexpression of cyclin D1. Studies have reported that IKKα up-regulates transcription of cyclin D in response to Wnt (41) or RANK signaling (20). Our results indicating increased cyclin D1 mRNA in IKKα−/− cells appear contradictory to the previous observation demonstrating IKKα as a positive transcriptional regulator of cyclin D1. Cao et al. (20) demonstrated that there is poor mammary gland development and inability to lactate in IKKαAA/AA mice. This resulted from decreased cyclin D1 expression in lobulo-alveolar cells due to the absence of IKKα-mediated RANK-induced NF-κB activation. Unlike in IKKα−/− mice, no other abnormalities were noted in these mice. These findings suggest that there are marked differences in functional consequences of a lack of IKKα versus carrying an IKKAA/AA allele. Our study was performed with IKKα null cells using primary MEF cells. It is possible that a lack of wild type IKKα in other cells could potentially affect several other pathways inducing cyclin D1 expression, accounting for increased expression of cyclin D1 in our study. Albanese et al. (41), while looking at the role of IKKα in inducing cyclin D1 expression mediated by Wnt signaling, noted decreased expression of cyclin D1 in serum-starved cells. Their results indicate that expression of cyclin D1 was marginally higher in IKKα−/− cells exposed to serum for 6 h. IKKα−/− cells used in their study were subjected to ST3 protocol, which is a process known to induce multiple genetic changes in these cells, including loss of several critical proteins involved in cell cycle regulation. The use of primary MEF cells by us and different experimental conditions could potentially explain the observed difference in amount of cyclin D1 in serum-starved cells. Increased expression of cyclin D1 both at the mRNA and protein level and significant reversal of these changes in IKKα−/− mice strongly favor the possibility that the observed alteration in cyclin D is directly related to lack of IKKα in IKKα−/− cells. Moreover data with siRNA-mediated inhibition of IKKα were consistent with data observed in IKKα−/− cells.

Our results demonstrate that increased accumulation of cyclin D1 in IKKα−/− cells results both from increased transcription and from increased stability of cyclin D1. More recently, IKKα was shown to inhibit NF-κB activation by increasing both the turnover of the NF-κB subunit Rel A and c-Rel and their removal from proinflammatory gene promoters in a specific cell type such as macrophage (55). Cyclin D1 is one of NF-κB-responsive genes. The absence of IKKα-induced negative regulation of the NF-κB pathway in IKKα−/− cells may explain the increased expression of cyclin D in these cells. Consistent with this possibility are our ChIP analysis results demonstrating increased association of p65 to cyclin D1 promoter under serum-starved conditions. However, other possible mechanisms need to be considered and remain to be studied at this point, including the role of other components of NF-κB pathway.

In this study, we demonstrate that IKKα-induced phosphorylation of cyclin D regulates the amount and subcellular localization of cyclin D1. Thus, our data and results from earlier studies suggest multiple layers of cyclin D regulation during the cell cycle. This work should provide important insights into early phases of cell cycle regulation and oncogenesis.

3 Y.-T. Kwak, R. Li, C. Becerra, D. Tripathy, E. Frenkel, and U. N. Verma, unpublished observation.

4 C. Albanese, personal communication.
