Negative Regulation of the RelA/p65 Transactivation Function by the Product of the DEK Proto-oncogene*

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NF-κB-mediated transcriptional activation is controlled at several levels including interaction with coregulatory proteins. To identify new proteins capable of modulating NF-κB-mediated activation, a cytoplasmic two-hybrid screen was performed using the p65 C-terminal transactivation domain as bait and identified the product of the DEK proto-oncogene. DEK is a ubiquitous nuclear protein that has been implicated in several types of cancer and autoimmune diseases. DEK appears to function in several nuclear processes including transcriptional repression and modulation of chromatin structure. Our data indicate that DEK functions as a transcriptional corepressor to repress NF-κB activity. DEK expression blocked p65-mediated activation of an NF-κB-dependent reporter gene and also inhibited TNFα-induced activation of the reporter gene. Chromatin Immunoprecipitation (ChIP) assays showed that DEK associates with the promoters of the NF-κB-regulated cIAP2 and IL-8 genes in unstimulated cells and dissociates from these promoters upon NF-κB binding in response to TNFα treatment. Moreover, the expression levels of an NF-κB-dependent reporter gene as well as the NF-κB-regulated Mcp-1 and IκBα genes is increased in DEK−/− cells compared with wild-type cells. ChIP assays on these promoters show enhanced and prolonged binding of p65 and increased recruitment of the P/CAF coactivator. Overall, these data provide further evidence that DEK functions to negatively regulate transcription.

NF-κB plays an important role in many cellular processes by regulating the expression of genes involved in immune and inflammatory responses, cell adhesion, cell cycle regulation, angiogenesis, and apoptosis (1, 2). In mammals the NF-κB family includes five members: RelA (referred to as p65 from here on), c-Rel, and RelB as well as p105 and p100, which are processed into the p50 and p52 subunits, respectively (2, 3). Each subunit includes a Rel homology domain (RHD) containing the nuclear localization sequence. In addition, p65, c-Rel, and RelB contain an acidic transactivation domain (TAD)3 at the C terminus. The most well studied and predominant form of NF-κB consists of a heterodimer composed of the p50 and p65 subunits (4). In unstimulated cells most NF-κB resides in the cytoplasm bound by a member of the IκB family of inhibitory proteins (2, 3). Upon stimulation, IκB is phosphorylated by the IκB kinase (IKK) complex, which targets IκB for polyubiquitination and degradation by the 26 S proteasome, allowing NF-κB to translocate to the nucleus to activate transcription of its target genes (2, 3).

Upon entry into the nucleus, NF-κB binds to the promoters of its target genes to induce high levels of transcription of these genes. Activation of transcription requires interaction with coactivator proteins, such as those that possess histone acetyltransferase (HAT) activity (3). HAT enzymes function to facilitate alteration of chromatin structure that allows recruitment of the basal transcription factors and RNA polymerase II to the promoter to initiate transcription. NF-κB interacts with several different HAT enzymes including the CREB-binding protein (CBP) and its homolog, p300 (5–7), the p300/CBP-associated factor (P/CAF) (7–9), and members of the SRC/p160 family (7–9). These HAT enzymes function to acetylate the N-terminal tails of the core histones as well as to acetylate the p50 and p65 subunits of NF-κB (8, 10, 11) to stimulate high levels of NF-κB-dependent gene expression.

NF-κB-dependent gene expression is also modulated through interaction with histone deacetylase (HDAC) enzymes including the Class I HDACs: HDAC1, HDAC2, HDAC3 (10, 12, 13), and the Class III HDAC, SIRT1 (14). These HDAC proteins function to negatively regulate NF-κB transcriptional activity by deacetylating the N-terminal tails of the core histones as well as by deacetylating the p50 and p65 subunits of NF-κB. In support of this, treatment of cells with specific HDAC inhibitors results in potentiation of TNFα-induced NF-κB activity (12, 13, 15, 16). This indicates that negative regulation of NF-κB activity through interaction with transcriptional corepressor proteins plays an important role in the overall control of NF-κB activity.

DEK is a 375-amino acid nuclear protein that is ubiquitously expressed in most mammalian cells (17, 18) and was initially

3 The abbreviations used are: TAD, transactivation domain; ChIP, chromatin immunoprecipitation assay; IL, interleukin; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; aa, amino acid(s); IFN, interferon; Stat, signal transducer and activator of transcription; AR, androgen receptor; TNF, tumor necrosis factor.
identified as a fusion protein with the nucleoporin CAN in a minority of acute myeloid leukemia (AML) patients (19). Expression of the dek gene is increased in multiple tumor cell types including bladder cancer, hepatocellular carcinoma, glioblastoma, melanoma, and various forms of leukemia (17, 20). DEK has also been implicated in multiple autoimmune diseases such as lupus and juvenile rheumatoid arthritis (21). Homology searches indicate that DEK is unrelated to any known family of proteins and contains no known enzymatic domains (18). Very little is known about the actual function of DEK, however several studies have implicated DEK in transcriptional regulation, regulation of HIV-2 replication (22), mRNA splicing (23), and chromatin remodeling (24). Much of the recent research on DEK has focused on its role in mediating changes in DNA structure. However, DEK can exist in a complex with the HDAC2 and Daxx transcriptional corepressor proteins (25), indicating a role for DEK in regulating transcription. In addition, DEK also can interact with the AP-2α transcription factor to enhance its ability to activate transcription (26).

Previously DEK was shown to interact with the pets (peri-ets) site within the HIV-2 promoter and that expression from this promoter is enhanced by disassociation of DEK (22, 27), suggesting DEK acts as a transcriptional repressor (27). DEK was recently shown to be acetylated by the P/CAF transcriptional coactivator protein within its N terminus resulting in a decreased affinity for DNA and accumulation in interchromatin granule clusters (IGCs) (28). Based on these results, it was proposed that coactivator-mediated acetylation of DEK displaces DEK from promoters to allow transcriptional activation (28).

We identified DEK as a p65-interacting protein through a cytoplasmic yeast two-hybrid screen utilizing the C-terminal TAD (aa 313–551) of the p65 subunit as the bait protein. Based on this, we sought to determine the potential role of DEK in regulating NF-κB-mediated gene expression. Our data confirm that DEK interacts with the p65 subunit of NF-κB and can repress NF-κB-dependent gene expression in a concentration-dependent manner in transient transfection reporter gene assays. ChIP analysis demonstrates that DEK associates with the NF-κB-regulated IL-8 and cIAP2 promoters in uninduced cells and dissociates in response to TNFα stimulation. In addition, quantitative real time PCR (QRT-PCR) shows that basal expression of the NF-κB-regulated Mcp-1 and IkBα genes is increased in fibroblasts isolated from dek−/− mice relative to cells from wild-type mice. Taken together, these data indicate that DEK can function as a transcriptional corepressor protein to negatively regulate NF-κB-dependent gene expression.

**MATERIALS AND METHODS**

**Cells, Plasmids, and Other Reagents**—HeLa and HEK293T cells were obtained from ATCC and maintained in DMEM-H (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and penicillin/streptomycin. Immortalized DEK+/+ and DEK−/− MEFs were isolated from E14.5 embryos and were maintained in DMEM-H (Invitrogen) with 10% bovine calf serum (HyClone Laboratories, Logan, UT) and penicillin/streptomycin. CMV-p65, 3×kB-luc, GAL4-p65, and 5×GAL4-luc have been described previously (12). The DEK expression plasmid (FLAG-DEK) was provided by D. Markovitz (University of Michigan). The human androgen receptor (AR) and GAL4-AR(N terminus) expression plasmids (29) and the PSA-luciferase reporter plasmid (30) were obtained from L. Shemshedini. CMV-p53 (31, 32) is originally from B. Vogelstein (Johns Hopkins University). p53ConA.luc is described in Ref. 33 and was obtained from W. Taylor (University of Toledo). The Stat5b-dependent luciferase reporter plasmid containing three repeats of an IFN-activated site element derived from the IRF-1 gene is described in Ref. 34, and the Stat5b expression plasmid is described in Ref. 35. Antibodies used in these studies include: p65 (Rockland Immunocas chemicals, Inc, Gilbertsville, PA), RNA Pol II large subunit and IkBα (Santa Cruz Biotechnology, Santa Cruz, CA), DEK (BD Biosciences Pharmingen, San Diego, CA), β-actin and P/CAF (Abcam Inc., Cambridge, MA), nonspecific mouse and rabbit IgG and anti-FLAG M2 antibody (Sigma-Aldrich). Recombinant human tumor necrosis factor α (TNFα) (BIOSOURCE, Camarillo, CA) was used at a final concentration of 10 ng/ml.

**Transient Transfection/Reporter Gene Assays**—Transient transfection/reporter gene assays were performed in 24-well plates with FuGene 6 (Roche Applied Science) according to the manufacturer’s recommendations. Cells were harvested 48 h after transfection, lysed with M-PER cell lysis buffer (Pierce) and assayed for luciferase activity using a LMax luminometer (Molecular Devices, Sunnyvale, CA). All experiments were performed a minimum of three times in triplicate. Data were normalized to total protein assayed.

**Coimmunoprecipitation and Western Blot Analysis**—For coimmunoprecipitations, the cells were lysed in cell lysis buffer (50 mM Tris, pH 7.5, 85 mM KCl, 0.5% Nonidet P-40 with protease inhibitors) for 10 min on ice and the intact nuclei/membrane fraction was pelleted by centrifugation at 3,300 × g for 5 min. The supernatant was discarded and the nuclei lysed in nuclear lysis/IP buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1.0% Triton X-100; 0.5% Nonidet P-40; 10 mM EDTA with protease inhibitors) for 10 min on ice. The soluble nuclear extract was recovered by centrifugation at 16,000 × g for 15 min and protein concentration determined using the Bio-Rad protein assay reagent. Immunoprecipitations were performed using equal amounts of nuclear extracts with the indicated antibodies and protein A/G-agarose (Santa Cruz Biotechnology). The immunoprecipitated complexes were washed four times with 500 μl of nuclear lysis/IP buffer and resuspended in 15 μl of 2× SDS-PAGE sample buffer. The samples were boiled for 3 min and the proteins separated on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore). Membranes were blocked in 1% BSA and probed with the indicated antibodies. Proteins were visualized using either anti-rabbit IgG-horseradish peroxidase or anti-mouse IgG-horseradish peroxidase (Promega) and PicoWest chemiluminescent reagent (Pierce) followed by exposure to film.

**Chromatin Immunoprecipitation (ChIP) Assays**—ChIP analysis was performed essentially as described (36). Cellular proteins and DNA were cross-linked by adding formaldehyde to the growth medium to a final concentration of 0.1%. Cells were harvested in cold phosphate-buffered saline and lysed in cell
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Isolation of Total Cellular RNA and Real-time PCR—Total cellular RNA was prepared using TRIzol reagent (Invitrogen) as recommended by the manufacturer. cDNA was generated from 2 μg of total RNA by reverse transcription with AMV reverse transcriptase (Promega) according to the manufacturer’s recommendations. Two microliters of cDNA was used as the template in a total reaction volume of 25 μl containing final concentrations of 1X IQ SYBR Green Super mix (Bio-Rad) and 600 nM of each forward and reverse primer. Primer sequences were: human cIAP2: forward, 5'-ATG TTT TGT TTG TTG TGT TGG TTG-3'; reverse, 5'-TCC CCA GCA GCA AGA GAC-3'; GAPDH: forward, 5'-CTG TGA GCT TGC TTT TCC-3'; reverse, 5'-GCC ACC ATG AGC TCC TTT CTA-3'. Expression levels were normalized to β-actin and expressed as relative expression. Data were read and collected on the Bio-Rad iCycler.

RESULTS

DEK Interacts with the p65 Subunit of NF-κB—A cytoplasmic yeast two-hybrid screen initially identified DEK as a potential protein that can interact with the C-terminal TAD of p65. To confirm the interaction between DEK and p65 HEK293T cells were transfected with the indicated plasmids and immunoprecipitations were performed (from equal amounts of nuclear extracts) with FLAG antibody. As shown in Fig. 1A, lane 1, p65 coimmunoprecipitated with FLAG-DEK from nuclear extracts of cells transfected with both DEK and p65. In addition, a weak but reproducible interaction was observed between the FLAG-DEK and endogenous p65 (Fig. 1A, lane 3). This weak interaction is likely caused by the low basal level of nuclear p65 in uninduced cells and may indicate that DEK inhibits the ability of this nuclear p65 to activate transcription. No nonspecific interactions were observed from control transfected cells (Fig. 1A, lanes 2 and 4), indicating the specificity of the DEK-p65 interaction. The lower panels (Fig. 1A) show the input extracts used for the immunoprecipitation, probed for both p65 (middle panel) and DEK (lower panel). Overall these results suggest that an interaction between p65 and DEK occurs in the nuclei of intact cells.

Endogenous DEK and p65 Interact in a TNFα-inducible Manner—To further confirm this interaction, coimmunoprecipitations were performed to demonstrate an interaction...
between endogenous DEK and endogenous p65. Nuclear extracts from cells that were treated with TNFα for 20 or 120 min (or no TNF) were immunoprecipitated with DEK antibody and the immunoprecipitates analyzed by immunoblotting. In untreated cells, a low level of nuclear p65 coprecipitated with DEK (Fig. 1B, lane 1). In contrast, there was a strong interaction between p65 and DEK detected after a 20-min TNFα treatment (Fig. 1B, lane 2). At the 120-min TNFα treatment, a reduced amount of p65 coprecipitated with DEK despite a similar level of nuclear p65 to the 20 min treatment, (Fig. 1B, lane 3). This decrease is possibly caused by the association of p65 with transcriptional coactivators or with IκB, both of which may displace DEK. Taken together, the data in Fig. 1 show that DEK and p65 interact and confirm our two-hybrid results.

DEK Represses p65-mediated Transcriptional Activation in a Concentration-dependent Manner—To determine the effect of DEK on p65-mediated transcriptional activation, HeLa and HEK293T cells were transiently transfected with the NF-kB reporter plasmid and the indicated plasmids. For CMV-F-DEK, lanes 3, 4, and 5 were transfected with 10, 25, and 50 ng of plasmid, respectively. Lanes 7 and 8 were transfected with 25 and 50 ng of the plasmid, respectively. HeLa cells were also transiently transfected with PSA-luc (B), p53-luc (C), and IRF-luc (D) and the indicated plasmids. In all cases, lanes 3, 4, and 5 were transfected with 10, 25, and 50 ng of plasmid, respectively. Cells were harvested 48 h after transfection and extracts assayed for luciferase activity. Luciferase activities were normalized to total protein assayed and expressed as the average RLU/μg of extract ± S.D. of a representative experiment performed in triplicate.

Repressive Effect of DEK Is Not Cell Type-specific—Similar experiments were also performed in HEK293T cells to show that the ability of DEK to repress p65-mediated transcription is not cell type-specific. HEK293T cells (Fig. 2A, right panel) were transfected as indicated and assayed for reporter gene activity 48-h post-transfection. Similar to the effects observed in HeLa cells, coexpression of DEK with p65 in HEK293T cells caused a concentration-dependent decrease in p65-mediated activation of reporter gene expression (Fig. 2A, lane 3–5 with lane 2). These results further demonstrate that DEK is able to repress p65-mediated transcriptional activation and that the effect is not cell type-specific.

DEK Represses the Ability of Other Transcription Factors to Activate Transcription—To determine if the ability of DEK to repress transcription is specific to p65 or if DEK is also able to
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repress the activity of other transcription factors, transient transfection reporter gene assays were performed using reporter genes driven by the androgen receptor (PSA-luc), p53 (p53-luc), and Stat5B (IRF-luc). These results show that DEK also is able to repress AR-driven PSA-luc reporter gene expression and p53-driven expression of the p53-dependent reporter gene in a concentration-dependent manner (Fig. 2, B and C). In addition, DEK was also able to modestly repress Stat5B-mediated expression of the IRF-luc reporter gene but only at the highest concentration of transfected plasmid (Fig. 2D, lane 5). These data indicate that similar to other transcriptional corepressors, the repressive effect mediated by DEK is more general and not specific to repression of p65-mediated transcription.

DEK Represses TNFα-induced NF-κB-dependent Transcriptional Activity—We next wanted to determine whether DEK could repress the ability of endogenous TNFα-induced NF-κB to activate expression of the κB-luc reporter gene. HeLa cells were transfected with the κB-luc reporter gene plasmid and either the vector control plasmid (CMV-FLAG) or increasing amounts of the DEK expression plasmid. About 40 h after the transfection, the cells were either left untreated or were treated for 6 h with TNFα (10 ng/ml), the cells harvested and extracts assayed for reporter gene activity. In the absence of DEK expression, TNFα treatment resulted in a 35-fold increase in reporter gene expression (Fig. 3). Transfection of 10 ng of the DEK plasmid had only a negligible effect on TNF-induced reporter gene activity (Fig. 3). However, increasing the amount of transfected DEK expression plasmid to 25 ng and 50 ng resulted in a ~50% (for 25 ng) and 90% (for 50 ng) reduction in TNFα-induced reporter gene activity (Fig. 3). In contrast, when a mutant κB-luc reporter plasmid was used, as expected there was no TNFα-induced increase in reporter gene activity, and DEK expression had no effect on reporter gene expression in the presence or absence of TNFα stimulation (data not shown). Because the NF-κB binding sites in this promoter are mutated to prevent binding of NF-κB, this indicates that the DEK-mediated repression is dependent on binding of NF-κB to the promoter.

DEK Represses NF-κB through the p65 Transactivation Domain—Based on the co-IP experiments as well as the yeast two-hybrid screen, our data indicate that DEK represses NF-κB-dependent gene expression by interacting directly with the C-terminal TAD of the p65 subunit. To directly determine if DEK represses NF-κB activity through the p65 TAD, we used a C-terminal region of p65 (aa 270–551) that includes the TAD fused to the GAL4 DNA binding domain (G4-DBD) in reporter gene assays. Expression of the G4-p65(TAD) fusion protein resulted in a high level of expression of the 5× GAL4-luc reporter gene (Fig. 4A, lane 2). Cotransfection of 10 ng of the DEK expression plasmid had a slight repressive effect on reporter gene expression (Fig. 4A, lane 3 versus lane 2). However, cotransfection of either 25 ng or 50 ng of the DEK expression plasmid resulted in an ~50 and 90% repression, respectively (Fig. 4A, lanes 4 and 5 versus lane 2) of G4-luc reporter gene activity. The DEK-mediated repression was dependent on the p65 TAD since no effect was seen when DEK was co-expressed with the GAL4 DBD (Fig. 4A). In addition, we also tested the effect of DEK on the activity of GAL4-p53 (Fig. 4B) and GAL4-AR(N terminus) (Fig. 4C) fusion proteins to activate transcription. Co-expression of DEK with GAL4-AR resulted in a strong repression of GAL4-AR activity (Fig. 4B). Similarly, coexpression of DEK with GAL4-p53 also resulted in strong repression of the GAL4-p53 fusion protein to activate expression of the reporter gene (Fig. 4C). Thus, similar to the data shown in Fig. 2, DEK can also repress the ability of these heter-

![FIGURE 3. DEK represses TNFα-induced NF-κB activity in a concentration-dependent manner. HeLa cells were transiently transfected with the indicated plasmids and either the vector control parental vector (CMV-F) or increasing amounts of the CMV-F-DEK plasmid. Approximately 40 h after transfection, the cells were either left untreated or were treated with TNFα (10 ng/ml) for 6 h. The cells harvested and extracts assayed for reporter gene activity. The absence of DEK expression, TNFα treatment resulted in a 35-fold increase in reporter gene expression (Fig. 3). Transfection of 10 ng of the DEK plasmid had only a negligible effect on TNF-induced reporter gene activity (Fig. 3). However, increasing the amount of transfected DEK expression plasmid to 25 ng and 50 ng resulted in a ~50% (for 25 ng) and 90% (for 50 ng) reduction in TNFα-induced reporter gene activity (Fig. 3). In contrast, when a mutant κB-luc reporter plasmid was used, as expected there was no TNFα-induced increase in reporter gene activity, and DEK expression had no effect on reporter gene expression in the presence or absence of TNFα stimulation (data not shown). Because the NF-κB binding sites in this promoter are mutated to prevent binding of NF-κB, this indicates that the DEK-mediated repression is dependent on binding of NF-κB to the promoter.](https://example.com/figure3.png)

![FIGURE 4. DEK inhibits NF-κB activity through the p65 transactivation domain. A. HeLa cells were transiently transfected with the indicated plasmids and the 5× GAL4-luc reporter plasmid. Extracts were assayed for luciferase activity ~48 h after the transfection. In addition, HeLa cells were transfected with a GAL4-p53 fusion (B) or a GAL4-AR(N terminus) fusion protein (C) and the indicated plasmids and extracts assayed for luciferase activity. Luciferase values were normalized to total amount of protein assayed and expressed as average RLU/μg of extract ± S.D. All experiments were performed in triplicate and performed three times with similar results.](https://example.com/figure4.png)
DEK Associates with the NF-κB-regulated cIAP2 and IL-8 Promoters—To determine if DEK is associated with the promoters of NF-κB-regulated genes, ChIP experiments were performed in which HeLa cells were untreated or treated with TNFα for the indicated times. After cross-linking of protein-DNA complexes, nuclear extracts were used in immunoprecipitations with the indicated antibodies and PCR performed using primers specific for the appropriate promoters on the immunoprecipitated DNA. DEK associated with both the cIAP2 and IL-8 promoters in untreated cells and remained associated with the promoters at the early TNFα time points (Fig. 5, UT: 30 min, lanes 1–3 for cIAP2; UT and 15 min lanes 6–7 for IL-8). DEK began to dissociate from the promoters by 60 min for cIAP2 (Fig. 5, lane 4) and 30 min for IL-8 (Fig. 5, lane 8). Most importantly, the dissociation of DEK from both promoters correlates with increased binding of p65 to these promoters (Fig. 5). The association of RNA Pol II with these promoters was used as a positive control. As predicted, neither DEK nor p65 were associated with the constitutively expressed GAPDH promoter (Fig. 5, lanes 11–15) and none of the promoters were precipitated with a nonspecific antibody (IgG) (Fig. 5).

To correlate the dissociation of DEK from the cIAP2 and IL-8 promoters with the TNFα-induced expression of these genes, expression was analyzed by real-time PCR analysis. For cIAP2, low levels of expression were observed in untreated cells and cells treated with TNFα for 15 and 30 min (Fig. 5B, left panel). However, as DEK began to dissociate from the promoter at 15 and 30 min (Fig. 5A), expression of cIAP2 gradually increased (Fig. 5B). At 60 min, where DEK binding is at its lowest level, cIAP2 expression is maximal (Fig. 5, A and B). After a 2-h TNFα treatment, DEK binding to the promoter is slightly higher (Fig. 5A) and expression is lower (Fig. 5B) compared with that at 60 min. A similar pattern is seen with IL-8 where maximal TNFα-induced expression is detected at 30 and 60 min (Fig. 5B, right panel) coinciding with the lowest level of DEK association with the promoter (Fig. 5A). Taken together, these data show that DEK associates with the promoters of two NF-κB-regulated genes (but not the constitutively expressed GAPDH gene) and that dissociation of DEK from these promoters in response to TNFα treatment correlates with increased expression of these genes.

The Absence of DEK Results in Increased Basal Expression of NF-κB-regulated Genes—To further analyze the role of DEK in repression of NF-κB-dependent gene expression we utilized immortalized fibroblasts from dek−/− knock-out mice and wild-type control litters. To analyze the effect of loss of DEK on NF-κB-dependent gene expression, wild type and DEK−/− cells were transiently transfected with the κB-luc reporter plasmid. About 36 h after the transfection, cells were either left untreated or treated with TNFα (10 ng/ml) for 6 h, harvested and assayed for luciferase activity. In the absence of TNFα treatment, basal reporter gene expression was 5.6-fold higher in the DEK−/− cells compared with the wild-type cells (Fig. 6A). TNFα treatment of the wild-type cells resulted in a 4.4-fold induction of reporter gene activity, whereas TNFα treatment of
the DEK−/− cells resulted in a 24-fold increase in reporter gene activity (Fig. 6A). Thus, in the absence of DEK, TNFα-induced NF-κB activity increases about 5-fold compared with wild-type cells, consistent with our observation that DEK represses the ability of NF-κB to activate transcription.

To analyze the effect of the absence of DEK on expression of endogenous, NF-κB-regulated genes in response to TNFα treatment, real-time PCR analysis was used to monitor the expression of Mcp-1 and IκBα. For both Mcp-1 and IκBα, basal expression was elevated in the DEK−/− cells compared with the wild-type control (Fig. 6B). A large increase in Mcp-1 expression with all four TNFα treatments was observed in the DEK−/− cells compared with the wild-type cells (Fig. 6B). For IκBα, in addition to increased basal expression in the DEK−/− cells, a greater than 2-fold increase in expression was also observed at the 15- and 30-min TNFα treatments, but only a negligible increase with the longer treatments (Fig. 6B). These data are in agreement with the reporter gene assays that show an increase in TNFα-inducible gene expression in the DEK−/− cells compared with the wild-type cells, further demonstrating a role for DEK in repressing NF-κB-dependent transcription. These results are also in agreement with our ChIP data showing that DEK associates with NF-κB-regulated promoters in untreated cells and dissociates from the promoters upon TNF treatment (see Fig. 5).

To further demonstrate the role of DEK in regulating expression of an NF-κB-regulated gene, IκBα protein expression was monitored in wild type and DEK−/− cells in response to TNFα treatment by immunoblotting. In the wild-type cells a typical pattern of IκBα degradation and resynthesis was observed (Fig. 6C, lanes 1–5). In the DEK−/− cells a basal level of IκBα was detected that was similar to wild-type cells and once again the typical pattern of degradation and resynthesis was observed (Fig. 6C, lanes 6–10). However, the amount of IκBα that was resynthesized in the DEK−/− cells was consistently greater at all of the time points compared with the wild-type cells (Fig. 6C, lanes 7–10 versus 2–5). These data correlate well with the increased level of IκBα expression observed by real-time PCR in the DEK−/− cells (Fig. 6B, lower panel). Taken together, these data further support a role for DEK in repressing NF-κB-dependent gene expression. It should also be noted that in both the immunoblotting and real-time PCR, expression of the NF-κB-regulated genes was compared with expression of the constitutively expressed β-actin. No detectable difference in β-actin expression (protein or mRNA) between the DEK−/− cells and the wild-type cells was observed, indicating that there may be some promoter specificity for DEK-mediated repression.

Effect of the Absence of DEK on Binding of p65 to the IκBα and Mcp-1 Promoters—To assess the mechanism by which DEK represses the ability of NF-κB to activate transcription, we per-
formed ChIP assays on the Mcp-1 and IκBα promoters in the wild-type and DEK−/− cells to determine if the absence of DEK affects binding of p65 to these promoters. The cells were untreated or treated with TNFα (10 ng/ml) for the indicated times and after cross-linking of protein-DNA complexes, immunoprecipitations were performed from nuclear extracts using an antibody specific for p65. Primers specific for the Mcp-1 and IκBα promoters were used to amplify immunoprecipitated DNA. In wild-type cells, a typical pattern of recruitment of p65 to both the Mcp-1 (Fig. 7, lanes 1–5) and IκBα (Fig. 7, lanes 11–15) promoters was observed in response to TNFα treatment with peak binding occurring at 30 min (Fig. 7, lanes 3 and 13) and a gradual decrease in binding at the 60 and 120 min (Fig. 7, lanes 4 and 5 and 14 and 15). Interestingly, in the absence of DEK, the TNFα-induced recruitment of p65 to both the Mcp-1 and the IκBα promoters was enhanced and prolonged (Fig. 7, lanes 6–10 versus lanes 1–5 and lanes 16–20 versus lanes 11–15).

The Absence of DEK Enhances P/CAF Recruitment to the Mcp-1 and IκBα Promoters—Previously it was shown that P/CAF-mediated acetylation of DEK results in decreased affinity of DEK for DNA (28), indicating a functional interaction between P/CAF and DEK in transcriptional regulation. We performed ChIP assays to determine if the absence of DEK affected the recruitment of P/CAF to the Mcp-1 and IκBα promoters. Recruitment of P/CAF to the Mcp-1 promoter in the wild-type cells was TNFα-inducible with maximal binding occurring at 60 and 120 min (Fig. 7, lanes 1–5). In the absence of DEK, recruitment of P/CAF is TNFα-inducible; however, P/CAF is recruited to the promoter at earlier times and at greater levels compared with the wild-type cells, correlating with the enhanced recruitment of p65 to the Mcp-1 promoter in the DEK−/− cells (Fig. 7, lanes 6–10). Similar to the Mcp-1 promoter, there was also enhanced association of P/CAF with the IκBα promoter. (Fig. 7, lanes 16–20 versus 11–15). Interestingly, both the Mcp-1 and IκBα promoters exhibited increased basal association of P/CAF in un-treated cells in the absence of DEK compared with the wild-type cells (Fig. 7, compare lane 1 and 6 and lane 11 and 16). This increase in P/CAF recruitment may contribute, at least in part, to the increased basal level of expression of the Mcp-1 and IκBα genes observed by real-time PCR (see Fig. 6B). Thus, the increased association of p65 and P/CAF with the Mcp-1 and IκBα promoters in the DEK−/− cells correlates well with the increased expression of these genes observed by real-time PCR analysis and further supports our hypothesis that DEK functions as a transcriptional corepressor protein.

**DISCUSSION**

Regulation of transcription by NF-κB requires a number of different proteins including transcriptional coactivator and corepressor proteins. In order to gain a better understanding of how NF-κB transcriptional activity is regulated we initiated a cytoplasmic yeast two-hybrid screen to identify proteins that interact with the C-terminal TAD of the p65 subunit. Previously, three proteins that interact with the p65 TAD were identified in transcription-based two-hybrid screens, however the bait proteins in these screens were deletion mutants that removed a significant portion of the TAD (37–39). This deletion was necessary because the p65 TAD can potently stimulate transcription in yeast. Interestingly, within the TAD are three LXXL (LXD) motifs (aa 436–441; 450–454; and 523–528). LXD motifs are frequently found in transcription factors as well as in transcriptional coactivator proteins and function to mediate protein-protein interactions (40, 41). For example, LXD motifs within the p160/SRC family of coactivators mediate the interaction between these proteins and the AF2 domain of ligand-bound nuclear receptors (42, 43). The three LXD motifs found within the p65 TAD appear to be important since mutation of any one of them results in a p65 protein that cannot activate transcription of a reporter gene in transient transfection reporter gene assays. Based on this, we felt it was necessary to utilize a bait protein that includes the entire TAD in order to identify new proteins that interact with and regulate the transcriptional activity of NF-κB. By using a cytoplasmic two-hybrid screen, we were able to use the intact TAD as our bait and were successful in identifying several proteins that can specifically interact with p65. In support of this strategy, another group used a similar C-terminal fragment from p65 as bait to identify the RING finger protein A07 as a transcriptional coactivator of NF-κB-mediated transcription (44).

One of the proteins we identified and the focus of the present study is the protein encoded by the DEK proto-oncogene. DEK is a nuclear protein that has been associated with a number of human diseases including autoimmune diseases and cancer (17, 18). Identification of the precise cellular function of DEK has been elusive since DEK does not belong to any characterized protein family and its similarity to other proteins is limited to...
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the 34-amino acid scaffold attachment (SAF) box (18), DEK has been proposed to be involved in a number of different nuclear processes including chromatin organization, mRNA processing, and transcription (18). Previously DEK was shown to bind to the peri-ets (pets) site in the HIV-2 promoter where it functions to repress transcription (22, 27). DEK also interacts with a protein complex that includes hDaxx and HDAC2 (25), further suggesting a role for DEK in transcriptional repression. Because we identified DEK in a two-hybrid screen as a protein that interacts with the C-terminal TAD of the p65 subunit of NF-κB, we wanted to determine if DEK could function to regulate the transcriptional activity of NF-κB. Our data strongly indicate that DEK functions as a transcriptional corepressor protein to repress the ability of NF-κB to activate transcription. We have confirmed the interaction between DEK and p65 by coimmunoprecipitations from transiently transfected cells as well as between endogenous DEK and p65. Transient overexpression of DEK results in a concentration-dependent repression of p65-mediated activation of a NF-κB-dependent reporter gene as well as TNFα-induced activation of the NF-κB-dependent reporter gene. In addition, DEK expression also resulted in repression of the ability of the androgen receptor, p53, and Stat5B to activate transcription indicating DEK functions as a general repressor of transcription. Consistent with the identification of DEK as a protein that interacts with the p65 C-terminal TAD, DEK was able to repress transcription of a heterologous fusion between the GAL4 DNA binding domain and the C-terminal TAD of p65. DEK was also able to repress the ability of the GAL4-AR (N-terminal domain) and GAL4-p53 fusion proteins to activate transcription.

Because the reporter gene assays relied on overexpression of DEK, which could result in nonspecific repression, we further confirmed that DEK functions as a transcriptional corepressor protein that can inhibit expression of NF-κB-regulated genes by using MEFs isolated from DEK knock-out mice or from wild-type littermates. Using quantitative real-time PCR we showed that the basal expression of two NF-κB-regulated genes (Mcp-1 and IκBα) was elevated in the DEK−/− cells compared with the wild-type cells. In addition, TNFα-induced expression was also elevated in the DEK−/− cells compared with the wild-type cells. Therefore, our data strongly indicate that DEK functions as a transcriptional corepressor protein to repress both basal and TNFα-induced NF-κB-dependent transcription through a direct interaction with the C-terminal TAD of the p65 subunit. Our observations in the DEK−/− cells strongly support our hypothesis that DEK has a specific function in transcriptional repression. Furthermore, we and others have shown that overexpression of other corepressor proteins, including HDAC1, HDAC2, HDAC3, and SMRT can repress NF-κB activity in transient transfection reporter gene assays (10, 12, 13, 45) and that HDAC1, HDAC3, and SMRT all directly interact with the p65 subunit of NF-κB (10, 12, 13, 45), in addition to directly interacting with many other transcription factors. The specific interaction of transcriptional coregulatory proteins with a large number of transcription factors is not uncommon. For example, the CBP/p300 interactome includes more than 300 physical or functional interactions with mammalian and viral proteins (46).

Using ChIP assays, we showed that DEK associates with the promoters of the IL-8 and cIAP2 genes in unstimulated HeLa cells. Upon TNFα stimulation DEK gradually dissociates from these promoters as binding of the p65 subunit of NF-κB to the promoters increases. Real-time PCR analysis showed that maximal expression of these genes was detected when the lowest level of DEK association with the promoters was detected. Thus it appears that at the early TNF time points a somewhat lower level of DEK remains associated with the promoters and functions to maintain a repressed or low level of expression of these genes. At the later time points most of the DEK dissociates from the promoters, likely correlating with maximal recruitment of coactivator proteins and resulting in maximal levels of gene expression. The fact that a low level of DEK remains associated with the promoters after TNFα treatment indicates that DEK may function to not only repress basal (uninduced) expression of these genes, but may also contribute to regulation of induced expression of these genes. This is further supported by the enhanced basal and TNFα-induced expression of both Mcp-1 and IκBα in the DEK−/− MEFs. In fact, several corepressors, including HDAC2, HDAC3, SMRT, and N-CoR remain associated with the IκBα promoter in response to TNFα induction (47). Moreover, coactivators (P/CAF and GCN5) and corepressors (HDAC1) can physically interact (48). Therefore, it is likely that many different corepressors are associated with promoters even under activation conditions, implying that these proteins function during activation, possibly to help maintain a proper level of transcription and to counteract the activity of coactivators.

The precise mechanism by which DEK represses transcription is not yet known. Our data indicate that DEK functions through a direct interaction with the p65 subunit of NF-κB. However it is also possible that DEK functions to repress transcription through other mechanisms including by binding directly to DNA. Our ChIP data show a high level of DEK association with both the cIAP2 and IL-8 promoters in unstimulated cells at a time when p65 association with these promoters is minimal, which may indicate a direct association of DEK with DNA. Another potential mechanism for DEK-mediated repression is through interactions with transcriptional coactivator proteins. A recent manuscript showed that DEK is able to repress the histone acetyltransferase activity of the P/CAF and p300 coactivators through a direct interaction with these proteins resulting in decreased levels of histone H3 and H4 acetylation (49). Interestingly, both P/CAF and p300/CBP are required for NF-κB-dependent transcriptional activation (5, 7, 50). Based on our coimmunoprecipitation experiments, DEK is associated with the low level of basal nuclear p65 in untreated cells. In this capacity DEK may function to repress the ability of this basally nuclear NF-κB to activate transcription. This may provide an important function to ensure that genes, such as those encoding proinflammatory cytokines are not expressed in the absence of an activating signal. In support of this, an increase in basal expression of both the Mcp-1 and IκBα genes is observed in untreated DEK−/− cells compared with the wild-type cells. Upon stimulation of cells with TNFα, there is an increase in the nuclear concentration of p65 and a subsequent increase in the amount of DEK associated with p65. Results
from ChIP assays show that DEK dissociates from the cIAP2 and IL-8 promoters in response to TNFα treatment and that this dissociation occurs as NF-κB binding to these promoters increases. Although DEK dissociates from these promoters, there is still some DEK that remains associated after TNFα stimulation indicating that DEK not only functions to repress basal expression of these genes but also contributes to repression of activated NF-κB. This is further supported by the higher level of expression of both the Mcp-1 and IκBα genes in the DEK<sup>−/−</sup> cells compared with the wild-type cells in response to TNFα treatment.

It has been well established that DEK is capable of binding directly to DNA (17, 18); however, it appears to bind DNA in a non-sequence specific, but structure-specific manner (51). This characteristic is similar to the DNA binding preference of the HMG family of non-histone proteins (18) that play a role in a wide variety of nuclear processes including regulation of transcription (52). The HMGI(Y) family plays an important role in regulation of the interferon-β (IFN-β) gene, which is regulated by the binding of NF-κB, members of the IRF family, and ATF-2/c-Jun to its promoter (53). Binding of these transcription factors recruits several coactivator proteins including CBP/p300 and P/CAM to the promoter resulting in the formation of an enhancerosome complex that includes the HMGI(Y) protein (53). In this context HMGI(Y) appears to function to mediate both enhancerosome assembly and disassembly (53). For example, acetylation of HMGI(Y) by P/CAM potentiates transcription of the IFN-β gene by stabilizing the enhancerosome whereas acetylation of a different lysine residue on HMGI(Y) by CBP destabilizes the enhancerosome leading to termination of IFN-β transcription (54). Much like the HMGI(Y) proteins, DEK may interact directly with DNA as well as with proteins bound to specific promoter elements. However, unlike the HMGI(Y) proteins, association of DEK with these promoters results in repression of transcription, possibly in association with corepressors such as HDAC2 (25) and/or through the inhibition of p300/CBP and P/CAM HAT activity (49). Interestingly, it was recently shown that P/CAM acetylates DEK at lysine residues within the first 70 amino acids of DEK resulting in decreased affinity of DEK for DNA and its accumulation in IGCs (28). Since P/CAM is required for NF-κB-dependent activation of transcription (7), it is possible that the recruitment of P/CAM to promoters by activated NF-κB results in the subsequent acetylation of DEK and its dissociation from the promoter. In support of this, we observed enhanced recruitment of p65 and P/CAM to the Mcp-1 and IκBα promoters in DEK<sup>−/−</sup> cells compared with the wild-type cells. Thus it appears that DEK-mediated repression is regulated, at least in part by coactivator-mediated acetylation of DEK and that DEK in turn can regulate the HAT activity associated with the p300/CBP and P/CAM coactivators.

Our data support previously published reports that DEK functions at least in part as a transcriptional corepressor protein, possibly through a direct interaction with DNA as well as in association with sequence-specific transcription factors such as NF-κB and with other transcriptional corepressor proteins such as HDAC2. Recent data demonstrating coactivator-mediated acetylation of DEK, resulting in its dissociation from DNA suggest a mechanism for overcoming DEK-mediated repression of transcription, which may in turn allow assembly of transcription factor-coactivator complexes to mediate activation of transcription. Future experiments will be directed at gaining a better understanding of the mechanism by which DEK functions as a transcriptional corepressor protein and how this contributes to regulation of transcription by NF-κB.

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