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Caspase-Dependent Apoptosis Induction by Targeted Expression of DEK in *Drosophila* Involves Histone Acetylation Inhibition

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Abstract As a nuclear phosphoprotein, proto-oncogene protein DEK is capable to changing chromatin structure. DEK was recently identified as an inhibitor of histone acetylation mediated by p300 and PCAF and to facilitate transcriptional repression. To elucidate the biological functions of DEK in vivo, we have constructed transgenic flies that overexpress the human DEK in the developing eye. Transgenic flies developed a severe rough eye phenotype, which is indicative of ectopically induced apoptosis. Genetic and biochemical analyses, including the rescue of the apoptotic phenotype by pan-caspase inhibitor protein p35 and caspase activity analyses, suggested that DEK induces apoptotic cell death through a caspases-9 and -3 dependent pathway. Using extracts from larval salivary glands, we have determined that the global histone acetylation levels of histone H3 Lys9 and H4 Lys5 were decreased upon DEK overexpression. Using chromatin immunoprecipitation assays, we have demonstrated that overexpression of DEK induced the histone H3 and H4 hypoacetylation of promoter of the antiapoptotic gene *bcl-2*. Co-expression of *bcl-2* also rescued apoptosis and the reduced expression of *bcl-2* gene was analyzed by real-time PCR. Our results indicate that acidic domain containing protein DEK might have a role in modulating both transcriptional regulation and apoptosis through HAT inhibitory activity. J. Cell. Biochem. 103: 1283–1293, 2008.

Key words: DEK; histone acetylation; *Drosophila*; apoptosis

The human DEK was originally identified in a fusion with the CAN nucleopore protein NUP214 by a specific chromosomal translocation (6;9) (p23;q34) in acute myeloid leukemia's (AML) [von Lindern et al., 1992]. DEK has been detected as an antigen involved in several human diseases, including systemic lupus erythematosus [Dong et al., 1998], juvenile rheumatoid arthritis [Sierakowska et al., 1993], and ataxia telangiectasia (ATM) [Meyn et al., 1993]. The proto-oncogene protein DEK has been reported to be over-expressed in a variety of neoplastic conditions [Kondoh et al., 1999; Larramendy et al., 2002]. It has also been reported that DEK is a protein that influences chromatin remodeling through the alteration of the chromatin topology [Alexiadis et al., 2000; Waldmann et al., 2002]. DEK could be involved in recruiting different proteins to chromatin such as transcriptional corepressor hDaxx and HDAC2 [Hollenbach et al., 2002], and to thereby influence transcriptional activity of target genes. DEK has also been found to be linked to the latency-associated nuclear antigen (LANA) through an interaction with MeCP2 in Kaposi's sarcoma-associated herpes virus latent infection [Krithivas et al., 2002].

The acetylation of nuclear core histones is thought to play important roles in various cellular functions. The biological importance of histone acetylation is correlated with the level of

Abbreviations used: HAT, histone acetyltransferase; UAS, upstream activating sequence Gal4-binding motif; FACS analysis, fluoresecence activated cell sorting analysis; TUNEL assay, terminal deoxynucleotidyl transferase dUTP nick end labeling assay.

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transcription [Strahl and Allis, 2000]. Histone H3 and H4 are hyperacetylated in active genes, whereas they are hypoacetylated in silent genes [Clarke et al., 1993; O'Neill and Turner, 1995; Grunstein, 1997]. We have previously reported on possible roles of the highly acidic domain containing protein DEK in the process of transcriptional regulation by demonstrating the p300 and PCAF-mediated HAT inhibitory activities by DEK [Ko et al., 2006]. In addition to DEK, another oncoprotein, designated SET/TAF-Ibeta, has also been shown to form a fusion protein with CAN in cases of undifferentiated leukemia [von Lindern et al., 1992]. SET/TAF-Ibeta is a subunit of the INHAT (inhibitor of acetyltransferases) complex, which targets histones and blocks histone acetylation by p300/CBP and PCAF [Seo et al., 2001].

It has been reported that SET/TAF-Ibeta and pp32, another INHAT subunit, have important roles in the regulation of apoptosis. Granzyme A that can activate apoptosis by inducing DNase, NM23-H1 and its inhibitor SET/TAF-Ibeta has been copurified [Fan et al., 2003]. Jiang et al. [2003] reported the induction of caspase-dependent apoptosis by pp32 and its homolog (PHAPs) and co-purification of another acidic protein prothymosin alpha as its inhibitor. Contrasting effects of SET/TAF-Ibeta as a positive regulator of apoptosis through the binding with Jcasp peptide in neuronal cells has been also reported [Madeira et al., 2005]. These findings indicate the roles played by proteins with highly acidic domains in regulation of both transcriptional and apoptotic cell death pathway [Chakravarti and Hong, 2003].

In order to investigate whether acidic domain containing protein DEK is involved in apoptosis pathway and if HAT inhibitory activity has a role in the process, we employed transgenic model that expresses DEK in *Drosophila*. The GAL4/UAS ectopic expression system has been an extremely valuable tool for the study of human disease by allowing the overexpression of human disease genes in a specific tissue or cell type of *Drosophila* [Brand and Perrimon, 1993]. For analysis of functional roles of DEK in vivo, we used transgenic *Drosophila* that expressed the human DEK gene in the developing eye which employs the *GMR-gal4* driver and provide the evidence of caspase-dependent apoptotic induction by DEK. Chromatin immunoprecipitation (ChIP) analysis demonstrates that DEK represses transcription of antiapoptotic gene *bcl-2* promoter via induction of histone H3 and H4 hypoacetylation and reduces Bcl-2 protein level. Together, our data suggest that possible regulatory role of DEK in apoptosis induction through the regulation of histone modification of target gene.

MATERIALS AND METHODS

Cloning and Plasmid Construction for DEK Overexpression

The coding sequence of DEK was PCR-amplified with ligation of either a 5′ *Bam*HI site-introduced primer (5′-CGCGGATCCGC-GATGTCCGCCTCGGCCCCTGCTGCG-3′, for pCMX PL1) and a 3′ *Eco*RI site-introduced primer (5′-CGCGAATTCGCGTCAAGAAATT-AGCTCTTTTAC-3′) or *Eco*RV site-linked primer (5′-CGCGATATCGCGTCAAGAAATTA-GCTCTTTTAC-3′, for pcDNA3.1). For the eukaryotic expression construct of DEK, PCR product was cloned into the cloning site of pCMX PL1 and pcDNA3.1 vector, respectively. Sequences of construct surrounding the cloning sites were verified by automated sequencing. For an analysis of the functional roles of human DEK in *Drosophila*, the forward (*Eag*I link-ATGTCCGCCTCGGCCCCTGC) and reverse (*Xba*I link-TCAAGAAATTAGCTCTT-TACA) primers were used to clone the coding region of the DEK gene by PCR. The product was gel-purified and subcloned into the *Eag*I–*Xba*I sites of the fly transgenic vector, *pUAST*.

Transgenic Fly Generation and Ectopic Expression of the Transgene

The germ-line mediated transformation was performed according to Rubin and Spradling [1982] and *pUAST-hDEK* transgenic lines were generated. Wild type w-, *GMR-gal4*, and *UAS-p35* were obtained from Bloomington stock center (Indiana University, Bloomington, IN), and *UAS-mbcl-2* was gift from Dr. Mignotte [Gaumer et al., 2000]. For ectopic expression of the transgene in the eye, DEK transgenic flies were crossed with the *GMR-gal4* driver line. To assess the affects of p35 overexpression on the DEK-mediated phenotype, *UAS-hDEK;UAS-p35*, and *UAS-hDEK;UAS-mbcl-2* transgenic flies were crossed with *GMR-gal4* driver line.

Cell Culture and Transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-

inactivated fetal bovine serum (Gibco) and 0.05% penicillin–streptomycin (Gibco) at 37°C with 5% CO_2 in humidified air. HeLa cells were seeded at 2×10^5cells/well in 6-well dish and transient transfection to HeLa cells were performed using lipofectamine 2000 (Invitrogen) with vector control pCMX PL1 (1 μg) and pCMX PL1-DEK (1 μg) for apoptosis assays.

FACS Analysis

To detect of early apopototic events, transfected cells were collected 72 h after transient transfection and washed twice in phosphate-buffered saline (PBS) and double-labeled with Annexin-V and propidium iodide (PI) by the Annexin-V-FLUOS kit (Roche Applied Science). The labeled cells were analyzed by a BD FACStation (BD Biosciences). The following instrumental settings were used to detect apoptosis: detector forward scatter, side scatter, fluorescence 1 for Annexin-V (FL1), fluorescence 3 for PI (FL3) and fluorescence emission of 10,000 cells was recorded. The results were analyzed with the FACScan Cell Quest software.

Caspase Assays

Caspases-3, 8, and 9 activities were measured by an Apoprobe-3, 8 caspases-3, 8 fluorescent assay kits (Peptron, Korea) and Caspase-Glo 9 assay kit (Promega), in which a fluorogenic synthetic peptide DEVD-AMC (caspase-3), IETD-AMC (caspase-8), and proluminescent containing LEHD (caspase-9) were used as a substrate. To measure of each caspase activity, cells were transfected with pCMX PL1 (1 μg), pCMX PL1-DEK (1 μg), pcDNA3.1 (1 μg), and pcDNA3.1-DEK (1 μg) eukaryotic expression plasmids. After transfection, cells were lysed and incubated with substrates for activated caspases-3, 8, and 9. Total proteins were extracted in adult flies and used for capase-3, 8, and 9 activities as well as HeLa cell lysates. The fluorescence of the released AMC was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm (Victor3, Perkin Elmer) and luminescence signal was measured in a plate-reading luminometer (FLUOstar Optima, BMG Labtech).

RT-PCR and Western Blot Analysis for the Expression of DEK in Transgenic Flies

Total RNA and total proteins were prepared from *GMR > hDEK* adult flies. For the RT-PCR, first stranded cDNA generated by AMV RTase (Roche) and cDNA was the subject of PCR amplification with the *DEK* primers. Western blots were performed with the anti-DEK antibody (BD Bioscience). The *rp49* gene and β-actin protein were used as controls.

Scanning Electron Microscopy

To assess the effect of DEK overexpression, *GMR > hDEK* flies anaesthetized, mounted on the stage and observed under Leo 1455VP Environmental Scanning Electron Microscope (KBSI, Korea) in the low vacuum mode.

Apoptosis Assay in Drosophila Eye Disc

Third instar larvae from *GMR > hDEK* were dissected in Drosophila Ringers' solution and eye imaginal discs were fixed. After washing, prepared samples were used in apoptosis assay. The TUNEL (TdT-mediated dUTP nick end labeling) reaction was carried out using an in situ Cell Death Detection Kit (Roche).

Western Blot Analysis

For western blot, proteins were extracted from salivary gland of flies and HeLa cells. Proteins were separated in 12–17% SDS–PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). After blocking with 5% skim milk in TBST (150 mM NaCl, 50 mM Tris-HCl, pH8.0, and 0.05% Tween 20) for 1 h, and then membranes were detected using the specific antibodies and the protein levels were detected horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies. The primary antibodies used for western blot were anti-DEK (BD Biosciences), anti-caspase-9 (R&D systems), anti-acetyl-histone H3 Lys9, anti-acetyl-histone H4 Lys5, anti-histone H3 (Upstate Biotechnology), anti-Bax, caspase-9 (active) (Santa Cruz Biotechnology) or anti-Bcl-2, Bcl-xl, caspase-8 (Cell Signaling Technology) antibodies. The antibody–antigen complexes detected using the ECL system (Santa Cruz Biotechnology). The band intensities in Western blot were quantitated by Quantity One Gel doc system version 4.6.2 (BioRad).

ChIP Analysis

The HeLa cells were transfected with 4.8 μg of DNA and harvested after 72 h. Cells were cross-linked with 1% formaldehyde in the medium for 10 min at 37°C, followed by the addition of 10× glycine for 5 min at room temperature, after

which they were scraped into SDS lysis buffer. The samples were further sonicated and diluted for immunoprecipitation with antibodies as indicated. Each 5 μg of anti-acetyl-histone H3 and anti-acetyl-histone H4 antibodies were employed for immunoprecipitation (Upstate Biotechnology). The immunoprecipitates were eluted and reverse cross-linked. The DNA fragments were purified and PCR amplified for quantification. The primers utilized for *bcl-2* promoter analysis were 5′-CCAGGCAGCTTAATACATTCTTTTTAG-3′ and 5′-TGATGCTGAAAGGTTAAAGAAAAAAC-3′ for Cdx and 5′-GTGTTCCGCGTGATTGAAGAC-3′ and 5′-CAGAGAAAGAAGAGGAGTTATAA-3′ for TATA promoter region. PCR products were separated by 1% TAE electrophoresis agarose gel and 20% polyacrylamide gel.

Quantitative RT-PCR for Bcl-2 Expression of Transient Transfected Cells

For quantitative RT-PCR analysis, total RNA was isolated from untreated, pCMX PL1 (1 μg), and pCMX PL1-DEK (1 μg) transfected cells using TRIZOL Reagent (Invitrogen). The first cDNA was synthesized by M-MLV reverse transcriptase (Promega) and generated cDNA was used for quantitative RT-PCR (ABI Prism 7900 Sequence Detection system, Applied Biosystems). mRNA levels were expressed as the relative fold change against the normalized *β-actin* mRNA. The comparative cycle threshold (Ct) method (User Bulletin 2, Applied Biosystems) was used to analyze the data. Experiments were repeated at least five times. Primers used in the quantitative RT-PCR were the following, *bcl-2*, 5′-AAGCTGTCGCAGAGGGGCTA-3′ (sense) and 5′-CTGGATCCAGGTGTGCAGGT-3′ (antisense); *β-actin*, 5′-CAAGAGATGGCCACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (antisense).

Statistical Analysis

Statistical analysis was carried out using the SPSS statistical software (release 10.0.5, SPSS, Inc.). Analysis of variance (one-way ANOVA) was used to determine differences among means.

RESULTS

DEK Induces Caspase-Dependent Apoptosis in *Drosophila*

To produce a *Drosophila* model to study DEK functions, we utilized GAL4/UAS system promoter/enhancer sequences that direct expression of DEK in a subset of cell types in the larval eye-antennal imaginal disc of *Drosophila*. The *UAS-hDEK* was constructed by cloning the full length DEK cDNA into the *pUAST* vector and transgenic lines were generated. The DEK expression of transgenic flies was confirmed by RT-PCR and Western blot with anti-DEK antibodies (Fig. 1A). The adult eye phenotypes of flies from transgenic lines and wild types were then compared. The adult *Drosophila* compound eye is composed by regularly spaced ommatidia with an ordered array of photoreceptor neurons and accessory cells (Fig. 1B, *WT*). Expression of DEK in the eye leads to a rough and disordered arrangement of ommatidia because of their irregular shapes and dimensions. The ommatidia lacked their regular hexagonal shape and appeared to be fused and missing bristles were obvious (Fig. 1B, *GMR > hDEK*). Next, we performed TUNEL assays by staining of the third instar larval eye discs with ectopic expression of DEK to confirm the possibility that a disrupted *Drosophia* eye phenotype is the result of apoptosis. When overexpressed, DEK induced a marked increase in the number of TUNEL positive cells compared with wild type eye disc (Fig. 1C, *GMR > hDEK*). In control eye antennal discs, relatively little apoptosis was observed (Fig. 1C, *WT*). To further verify whether DEK induced apoptosis is associated with activation of caspase-3, the enzymatic activity of caspase-3 in *Drosophila* extracts was also measured. Overexpression of DEK augmented caspase-3 activity compared with *GMR-gal4* and *UAS-hDEK* expressed cell extracts (Fig. 1D). Furthermore, we found that the caspase-3 activity was suppressed substantially when the caspase-3 inhibitor Ac-DEVD-CHO was treated (Fig. 1D, *GMR > hDEK + inhibitor*). These results indicate that the overexpression of DEK triggers apoptosis which is facilitated through the caspase-3 activation pathway.

The baculovirus origin caspase inhibitor p35 gene encoding the 35 kDa protein is a cell survival protein that functions as an inhibitor of virus induced apoptosis in insect cells [Clem et al., 1991]. The inhibitor also functions in *Drosophila*, and the expression of p35 in *Drosophila* eliminates most, if not all, normally occurring cell death in the developing embryo

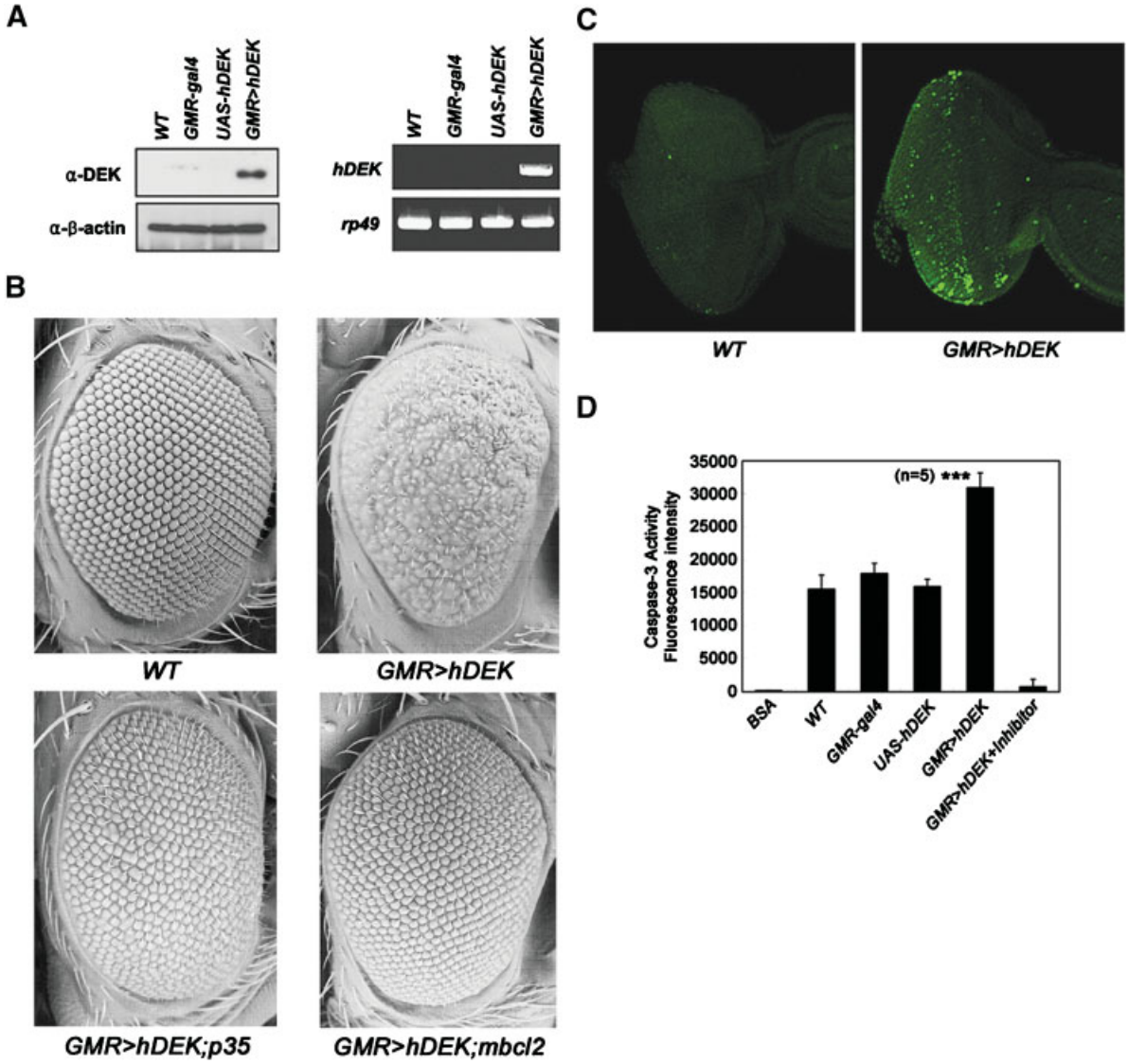

Fig. 1. DEK induces caspase-dependent apoptosis in *Drosophila*. **A**: Tissue specific DEK over-expression was confirmed by Western blot (**left**) and RT-PCR (**right**). Equal loading of samples were detected by anti-β-actin and *rp49* specific primer. **B**: Ectopic expression of DEK in eye of *Drosophila* induced by eye-specific driver *GMR-gal4*. Adult eyes from transgenic flies were viewed by scanning electron microscopy (SEM). Tested flies carried a transgene expressing the GAL4 under the control of GMR. Wild-type fly (upper left) did not overexpressed DEK, whereas *GMR > hDEK* (*GMR-gal4/+; UAS-hDEK*, upper right) overexpressed DEK. *GMR > hDEK;p35* (lower left) and *GMR > hDEK;mbcl-2* (lower right) overexpressed DEK, p35 and mbcl-2. **C**: Wild-type (left) as a control and *GMR > hDEK* (right) third instar larvae eye-antennal discs dissecting and labeled with fluorescein-dUTP and terminal deoxynucleotidyl transferase (TdT) using TUNEL assay for DNA fragmentation in the apoptotic cells. **D**: The enzymatic activities of caspase-3 were measured using total proteins from negative control group (*Wild-type*, *GMR-gal4*, and *UAS-hDEK*), and DEK overexpression (*GMR > hDEK*) flies. Significance as compared to wild-type flies (statistical difference determined by ANOVA, $***P < 0.001$). Error bars represent the SD.

and eye [Hay et al., 1994]. To determine whether phenotype observed in *Drosophila* eye by DEK overexpression is indeed the result of apoptotic cell death, we examined whether the eye morphology phenotype is altered by the presence of the baculoviral antiapoptotic protein p35. Co-expression of p35 and DEK using the GMR promoter rescued the apoptotic phenotype significantly (Fig. 1B, *GMR > hDEK;p35*). This result is consistent with our TUNEL and caspase analysis that suggests the phenotypic defects we observed are due in large part to cells dying through the apoptosis.

DEK Induces Caspase-Dependent Cell Death in HeLa Cells

To determine whether overexpression of DEK can induce apoptosis in HeLa cells, CMX PL1-DEK transfected cells were labeled with Annexin-V and propidium iodide and cell death in CMX PL1-DEK transfected cells were quantitated by flow cytometry. Values of apoptotic cells were represented as bar graph on lower right panel of Figure 2A. Compare to the vector transfected cells, there were around 9.2% increase in apoptotic cell death in CMX PL1-DEK treated cells (Fig. 2A). Expression of DEK in HeLa cells transfected with CMX PL1-DEK was confirmed by western blot and RT-PCR (Fig. 2A, lower left panel). The bottom panel represents RT-PCR of β-actin. Among the caspases, caspase-3 has been suggested to be an important mediator of apoptosis in response to multiple signals [Thornberry and Lazebnik, 1998]. Caspase-3 activities of both DEK and control vector transfected cells were assayed by measuring the degradation of the fluorometric peptide DEVD-AMC. The activity of caspase-3 was marginally increased by the transfection of the control pcDNA3.1 vector and further activation was observed with pcDNA3.1-DEK overexpression (Fig. 2Bi). Consistent results were obtained when CMX-PL1-DEK clones were used, only slightly higher CMX-PL1 control vector cell death effect than that of pcDNA3.1 (data not shown). Next, we tested whether DEK overexpression induce any initiator caspases such as caspase-8 and caspase-9 processing, which trigger subsequent caspase-3 induction. There was no caspase-8 induction when CMX PL1-DEK was transfected compare to those of untreated and CMX PL1 vector transfected cells with pro-caspase-8 level remained same with basal level of cleaved caspase-8 (p43/p41) when DEK was over-expressed (Fig. 2Bii,C, left panel). On the contrary, CMX PL1-DEK transfected cells showed substantially increased caspase-9 activity and decreased pro-caspase-9 and increased active caspase-9 (p35) levels, respectively (Fig. 2Biii,C, right panel) compare to those of untreated and CMX PL1 vector transfected HeLa cells suggest caspase-9 initiated apoptotic pathway. These results suggest that overexpression of DEK induces caspases-9 and -3 dependent cell death in certain fraction of HeLa cells.

DEK Induces Chromatin Hypoacetylation of the *Bcl-2* Promoter

Previous report that DEK could function as a repressive regulator of transcription by inhibiting histone acetylation suggested that HAT inhibitory activity of DEK might be related to apoptotic induction in this study. Using protein extracts for larval salivary glands of DEK overexpression mutants and control larvae, we compared the global levels of core histone actylations. The levels of acetylation at lysine 9 of histone H3 (AcH3K9) and at lysine 5 of histone H4 (AcH4K5), which are both hallmarks of active chromatin were all decreased after DEK overexpression following heat-treatment compared to that of Gal4 expressed control (Fig. 3A). Constant protein levels of histone H3 and β-actin were shown by western blot. These results are consistent with our previous study that DEK reduces histone acetylation level in vivo and promotes transcriptional repression when overexpressed [Ko et al., 2006].

Next, we tested the hypothesis that HAT inhibitory activity of DEK affect apoptotic

Fig. 2. Overexpression of DEK induces caspase-dependent cell death in HeLa. **A**: Flow cytometric analysis of HeLa cells double-labeled with Annexin-V and PI. Representative dot plots of control cell (**left panel**), CMX PL1 vector control (**middle panel**), and DEK overexpressed cell (**right panel**). In each of the plots, the lower left quadrants indicate viable cells that are negative for both Annexin-V binding and PI uptake; the lower right quadrants include apoptotic cells (Annexin-V-positive/PI-negative). Cells in the upper left and right quadrants correspond to live necrotic (Annexin-V-negative/PI-positive) and damaged (Annexin-V-positive/PI-positive) cells, respectively. Significance as compared to vector transfected cells (statistical difference determined by ANOVA, $*P < 0.05$). Error bars represent SD. The bar graphs represent average of five independent assays. DEK overexpression in the HeLa cells was confirmed by RT-PCR and Western blot. **Bi**: For measurements of caspase-3 activity, HeLa cells were transfected with pcDNA3.1 control vector and pcDNA3.1-DEK. After 72 h of transfection, cells were lysed, and the caspase-3 activities in the lysates were measured using substrate (2 μl of 2.5 mM). The mixture was incubated at 37°C for 30 min and activity was measured by fluorometer. Significance as compared to control vector transfected cells (statistical difference determined by ANOVA, $***P < 0.001$). **ii**: After transient transfection, cells were lysed and the caspase-8 activities in the lysates were measured using substrate (2 μl of 2.5 mM). **iii**: For measurements of caspase-9 activity, transfected cells were incubated with lumenogenic substrate. Following incubation, luminescence was measured using lumenometer ($***P < 0.001$). Activity is expressed as fold increase relative to the untreated control. Error bars represent SD for three independent experiments (Bi,ii) and SE for five independent experiments (Biii). Transient transfected cells were lysed and separated by 12% SDS–PAGE and Western blotted against anti-caspase-8, anti-pro- and cleaved caspase-9 antibodies, and as a loading control, β-actin antibodies (**C**).

pathway with ChIP analysis. The *bcl-2* family of genes appears to be important in the regulation of apoptosis. Members of this family are cellular homologues that are either proapoptotic or antiapoptotic [Cory et al., 2003]. The mechanism by which Bcl-2 prevents apoptosis by blocking cytochrome *c* release from mitochondria has been reported [Cory et al., 2003]. In

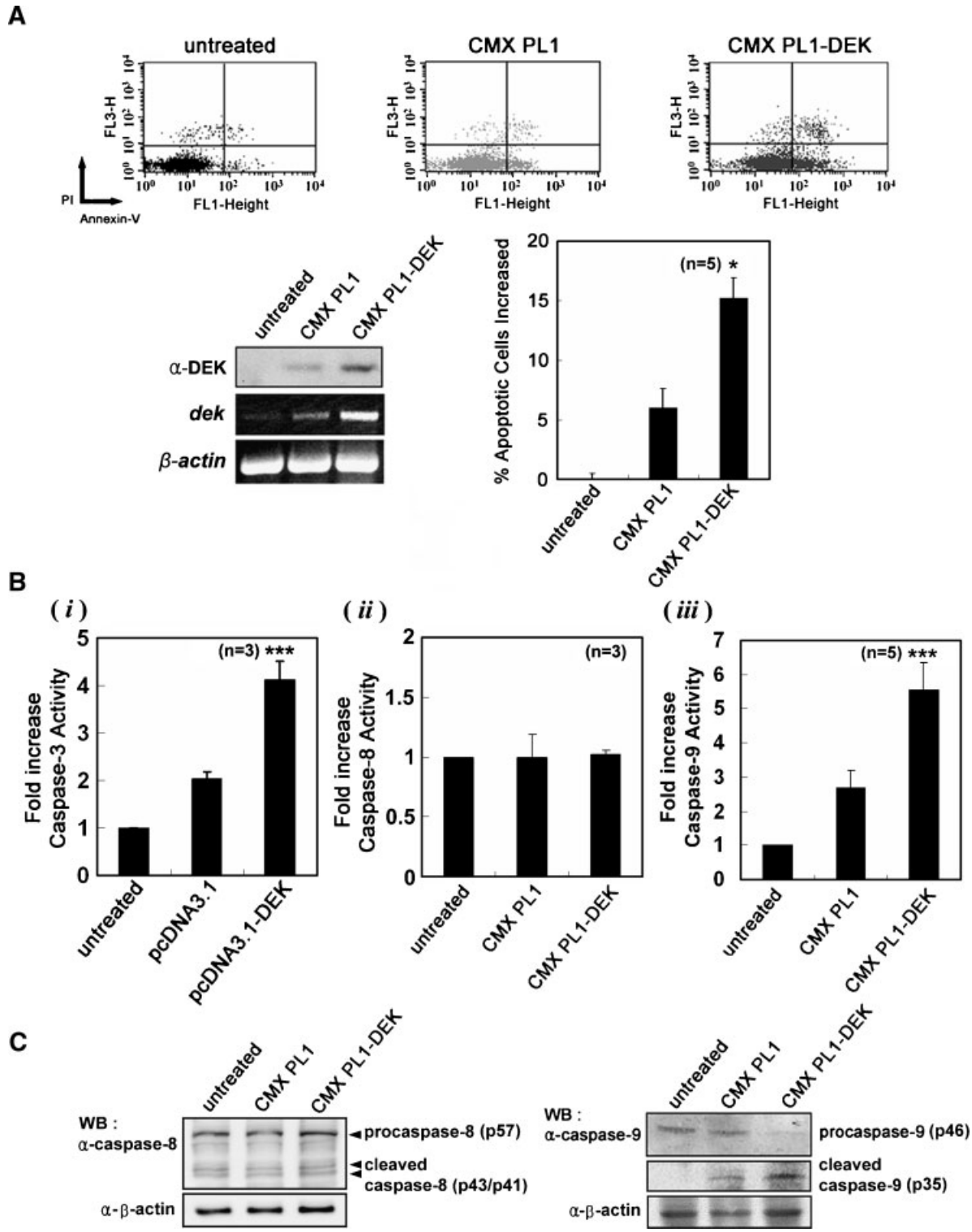

Fig. 2.

order to characterize the mechanism underlying DEK-mediated apoptosis and transcriptional regulation, we conducted ChIP assays with anti-acetylated histone H3 and H4 antibodies, and compared the levels of histone acetylation in antiapoptotic gene *bcl-2* promoter. With a constant level of input DNA from HeLa cells transfected with either CMX PL1 or

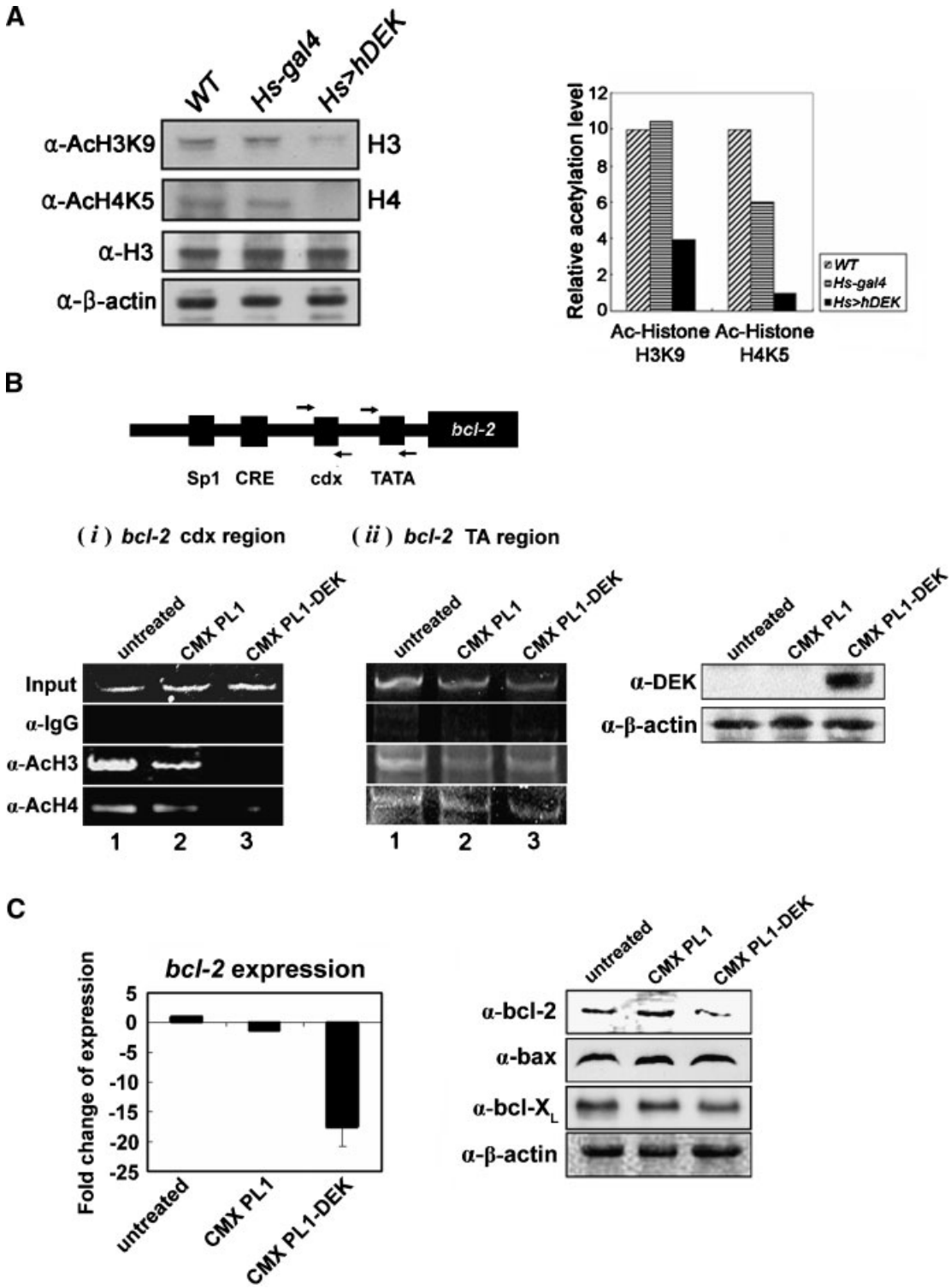

Fig. 3.

CMX PL1-DEK (Fig. 3Bi,ii, lanes 2 and 3). Mouse IgG was used for negative control. To examine the specificity of histone hypoacetylation by DEK, two different *bcl-2* promoter regions were compared for their histone acetylation levels by ChIP analysis. Interestingly, we noted a substantial decrease in the level of histone H3 and H4 acetylation of *bcl-2* Cdx promoter region, in the presence of CMX PL1-DEK (Fig. 3Bi, compare lanes 2 and 3). On the contrary, there were no change in histone H3 and H4 acetylation levels in TATA promoter region (Fig. 3Bii). On the basis of these results, we provide the evidence that the repressed transcription of antiapoptotic target gene *bcl-2* is caused by reduced histone acetylation by DEK and partially responsible for the induction of apoptotic cell death. Then, we quantified mRNA expression of *bcl-2* in HeLa cells transfected with CMX PL1-DEK and CMX PL1 vector by RT-PCR. DEK transfected cells significantly reduced *bcl-2* expression compare to those of CMX PL1 vector and untreated cell extracts (Fig. 3C, left panel). In order to analyze the DEK mediated decrease in Bcl-2 protein level, we transfected cells with CMX PL1-DEK and checked protein level of Bcl-2. We confirmed by immunoblot analysis that DEK expression significantly reduced Bcl-2 protein expression compare to that of untreated and control vector transfected cells (Fig. 3C, right panel). We further investigated whether another anti-apoptotic protein Bcl-xl is induced by DEK and found no differences in the expression level which indicated the possible bcl-2 specific down-regulation by DEK (Fig. 3C, right panel). Next, we investigated whether one of the Bcl-2 related family and pro-apoptotic gene *bax* might be activated during the DEK mediated apoptosis. Immunoblotting analysis indicated that the expression level of Bax remained unchanged when DEK was over-expressed (Fig. 3C, right panel). ChIP analysis for bax was also performed and there were no change in both histone H3 and H4 acetylation levels at *bax* promoter region (data not shown). These results indicate that both Bcl-xl and Bax are not involved in DEK induced apoptotic cell death.

To further investigate whether transcriptional repression of *bcl-2* by DEK is indeed responsible for the apoptosis induced by DEK, we performed rescue experiment using P[UAS-*dek*] and P[UAS-*bcl-2*] fly lines. Previous work has indicated that *bcl-2* expression is sufficient to counteract both developmental and X-ray induced cell death in *Drosophila* embryos [Gaumer et al., 2000]. Using transgenic flies carrying a human *bcl-2* cDNA placed under the control of UAS sequences, we coexpressed *bcl-2* and *dek* in *Drosophila* eye. The apoptotic eye phenotype was rescued significantly, thereby indicating that *bcl-2* expression can counteract the cell death induced by expression of *dek* in *Drosophila* (Fig. 1B, *GMR > hDEK;mbcl-2*). These results together demonstrate that DEK is involved in transcriptional regulation of antiapoptotic gene *bcl-2* via chromatin hypoacetylation.

DISCUSSION

In this study, we investigated the possibility that DEK activity might include the induction of caspase mediated apoptosis through regulatory activity toward histone modification to control transcription of apoptosis related target genes. First, we showed that overexpression of DEK causes disruption of ommatidia in eyes of *GMR-DEK* transgenic lines thereby indicating typical apoptosis induction. These data suggest that DEK may play a role in the apoptotic cell death process in *Drosophila*. Because the disruption of rough-eye phenotype was rescued by co-expression of p35, we assume the effects of DEK overexpression include that it directly regulates apoptosis and/or cell proliferation. Caspase-3

Fig. 3. DEK reduces histone acetylation and causes chromatin hypoacetylation in *bcl-2* promoter and reduces Bcl-2 protein level. **A**: Reduced histone acetylation after overexpression of DEK in *Drosophila*. Protein extract from wild-type control and heat-treated *Hs-gal4* flies and heat-treated *Hs > hDEK* mutants were separated by 17% SDS–PAGE and Western blotted against both anti-AcH3K9 and anti-AcH4K5 antibodies. The equal amount of sample loading was confirmed by Western blot with antibodies against β-actin or histone H3. Western blot analyses were presented quantitatively in bar graph (**A**, right graph). **Bi**,**ii**: The HeLa cells were transfected with the CMX PL1 and CMX PL1-DEK constructs. Following transfection, ChIP analysis using control IgG, anti-AcH3, and anti-AcH4 antibodies were performed. The immunoprecipitated DNA fragments were amplified by PCR from the Cdx (i) and TATA promoter (ii) regions of the *bcl-2* gene. DEK overexpression in transfected cells was confirmed by Western blot. **C**: Comparison of the relative gene expression levels of *bcl-2* mRNA in untreated, CMX-PL1 vector, and CMX-PL1-DEK transfected cells by quantitative RT-PCR (**left panel**). Western blot analysis was performed with the cell extracts of untreated, CMX-PL1 vector, and CMX-PL1-DEK transfected cells using anti-Bcl-2, anti-Bax, and anti-Bcl-xl antibodies, and as a loading control, β-actin antibodies (**right panel**).

activation was detected in both *Drosophila* and HeLa cells. Furthermore, induction of caspase-9 but not caspase-8 in DEK overexpressed cells indicates the possible cytochrome *c* dependent apoptotic pathway by DEK.

Next, we investigated the possible link between HAT inhibitory activity and apoptotic cell death by DEK. The DEK protein has at least three highly acidic amino acid domains and it is interesting that proteins with highly acidic domain (such as SET/TAF-Ibeta, pp32, and prothymosin-alpha) have important roles in both caspase-dependent and caspase-independent apoptosis regulation [Fan et al., 2003; Jiang et al., 2003]. Recent study has shown the evidence of a close relationship between transcriptional repression by blocking acetylation and phosphorylation of histones and apoptosis induction through initiating caspase induction by the acidic domain containing protein pp32 [Fan et al., 2006]. In this study, a possible mechanism of apoptosis induction by DEK was presented by promoting targeted histone hypoacetylation in promoter of antiapoptotic gene *bcl-2* and eventually induces caspase induction. The transcriptional repression of antiapoptotic gene *bcl-2* by DEK was further supported by the evidence that apoptotic phenotype of *Drosophila* eye was rescued when *bcl-2* was coexpressed.

Recent report suggests that cell death in response to DEK depletion was accompanied by increased protein stability of the p53 tumor suppressor at a post-transcriptional level [Wise-Draper et al., 2006]. However, we should not rule out the possibility that DEK could regulate apoptosis related target genes at transcriptional level, too. One of the possible mechanisms is that transcriptional repression of antiapoptotic gene *bcl-2* through mediating chromatin hypoacetylation by DEK. The fact that hypoacetylation of H3 and H4 at *bcl-2* promoter by DEK raises the question of whether the HDACs are involved in this process. No apparent changes in both histone acetylation levels in pro-apoptotic gene *bax* promoter region and protein expression level suggest that other pro-apoptotic factor(s) might be involved in DEK mediated apoptosis induction.

Although we have previously reported the transcriptional repression activity of DEK through HAT inhibition, there has been a report that DEK can act as a transcriptional activator [Campillos et al., 2003]. Because of such dual characteristics of DEK, elucidation of either apoptotic or antiapoptotic pathway in response to DEK according to the particular cellular environment and identification of mediators involved in both processes may further provide important information about physiological role played by DEK. Interestingly, the fact that p53 is necessary to maintain histone acetylation levels in *Drosophila* further suggests the possible connections among DEK, p53, and histone acetylation [Rebollar et al., 2006].

In summary, identification of the link between histone modifications, in particular, inhibition of histone acetylation and the induction of apoptotic cell death by DEK would provide important connection between two major cellular pathways. Also, our results indicate that DEK might have a role similar to the recently identified functions in modulating both transcriptional regulation and apoptosis through acidic domain containing proteins such as pp32 and SET/TAF-Ibeta.

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