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Proteomic Analysis of Apoptosis Related Proteins Regulated by Proto-Oncogene Protein DEK

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ABSTRACT

A nuclear phosphoprotein, DEK, is implicated in certain human diseases, such as leukemia and antoimmune disorders, and a major component of metazoan chromatin. Basically as a modulator of chromatin structure, it can involve in various DNA and RNA-dependent processes and function as either an activator or repressor. Despite of numerous efforts to suggest the biological role of DEK, direct target proteins of DEK in different physiological status remains elusive. To investigate if DEK protein triggers the changes in certain protein networks, DEK was knocked down at both types of cell clones using siRNA expression. Here we provide a catalogue of proteome profiles in total cell lysates derived from normal HeLa and DEK knock-down HeLa cells and a good in vitro model system for dissecting the protein networks due to this proto-oncogenic DEK protein. In this biological context, we compared total proteome changes by the combined methods of two-dimensional gel electrophoresis, quantitative image analysis and MALDI-TOF MS analysis. There were a large number of targets for DEK, which were differentially expressed in DEK knock-down cells and consisted of 58 proteins (41 up-regulated and 17 down-regulated) differentially regulated expression was further confirmed for some subsets of candidates by Western blot analysis using specific antibodies. In the identified 58 spots, 16% of proteins are known to be associated with apoptosis. Among others, we identified apoptosis related proteins such as Annexins, Enolase1, Lamin A, and Glutathione-*S*-transferase omega 1. These results are consistent with recent studies indicating the crucial role of DEK in apoptosis pathway. We further demonstrated by ChIP analysis that knock-down of DEK caused hyperacetylation of histones around Prx VI promoter which is upregulated in our profile. Using immunoblotting analysis, we have demonstrated the modulation of other caspase-dependent apoptosis related proteins by DEK knock-down and further implicate its role in apoptosis pathway. J. Cell. Biochem. 106: 1048–1059, 2009.

KEY WORDS: PROTEOMICS; 2-DE; DEK; APOPTOSIS

The DEK protein, known as an abundant 43 kDa nuclear phosphoprotein, has a strong association with critical human malignancies since this gene was originally identified in the t(6;9)(p22;q34) chromosomal translocation in a subset of acute myeloid leukemia (AML) [von Lindern et al., 1990]. Although the function and mechanisms of this fused protein leading to malignant

Abbreviations Used: HAT, histone acetyltransferase; HDAC, histone deacetylase; ENO1, enolase-1; GSTO1, glutathione-*S*-transferase omega 1; ANX5, annexin V; GRP78, glucose-regulated protein 78 kDa; PHB, prohibitin; Prx2, peroxiredoxin 2; PrxVI, peroxiredoxin VI; VCP, valosin-containing protein.

Dong-Wook Kim and Jung-IL Chae contributed equally to this work.

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transformation remain unexplored, it was reported that gene products from this chromosomal translocations often interfere with regulatory mechanisms controlling growth, differentiation, and survival of normal cells. Also previously reported relationship between high level of DEK transcript and proliferartion [Ageberg et al., 2006], together with the elevated transcripts of DEK associated with human tumors such as AML, hepatocellular carcinoma, glioblastoma, and melanoma [Kondoh et al., 1999; Grottke et al., 2000; Kroes et al., 2000; Larramendy et al., 2002; Casas et al., 2003; Carro et al., 2006], support a potential role of DEK in tumorigenesis. In addition, some evidences revealed that the DEK has a role in chromatin remodeling, through the alteration of the topology of chromatin [Waldmann et al., 2002]. Basically as a modulator of chromatin structure, it can involve in various DNA and RNA-dependent processes and function as either an activator or repressor. DEK is a subunit of a multiprotein transcriptional repressor complex, which includes hDaxx and HDAC2 [Hollenbach et al., 2002]. We have reported that p300- and PCAF-mediated histone acetyltransferase (HAT) inhibitory activities of DEK through its acidic domain containing regions [Ko et al., 2006]. Recent report suggested that DEK is a target gene of histone demethylase, LSD1 and repressed when LSD1 is knocked down [Scoumanne and Chen, 2007]. The involvement of DEK in various signal transduction pathway have been implicated including the regulation of apoptosis through HAT inhibitory activity and DNA damage repair through ADP-ribosylation [Kappes et al., 2008; Lee et al., 2008].

Apoptosis is the primary mechanism by which the body gets rid of genetically defective cells and is critical for preventing the accumulation of cells with tumorigenic potential. Changes in apoptotic regulation are considered to have a pivotal role in the malignant transformation. This is often initiated in cells with dysregulated growth to avoid the emergence of malignant clone. The induction of apoptosis often involved the activation of p53. Recently, Wise-Draper et al. [2006] suggested a novel role for DEK in cellular survival, involving the destabilization of p53 in a manner, which is likely to contribute to human carcinogenesis. DEK expression protects both HPV-positive cancer and primary human cells from apoptotic cell death. Cell death in response to DEK depletion was accompanied by increased protein stability and transcriptional activity of the p53 tumor suppressor and consequent up-regulation of known p53 target genes such as $p21^{CIP}$ and Bax. Consistent with a possible role for p53 in DEK-mediated cell death inhibition, the p53-negative human osteosarcoma cell line SAOS-2 was resistant to the knock down of DEK.

Proteomics is an ideal technology for detecting changes in protein expression, as it allows comparison of two or more samples at a relatively global level, without prior knowledge of the pathways influenced by the experimental manipulations [Blackstock and Weir, 1999]. We have used 2-DE, high-throughput image analysis, and candidate picking, followed by MALDI-TOF and/or MALDI-TOF/TOF MS to investigate changes in the proteome profile due to the knocking down of DEK proteins. This is a powerful technique for discriminating the proteome profile changes in part due to the advance of knowledge in protein separation and of recent development of highly sensitive MS techniques. We have demonstrated the protein expression profile modulated by DEK knock-down and especially have shown that DEK is able to regulate certain numbers of proteins play major roles in apoptosis pathway.

MATERIALS AND METHODS

MATERIALS

IPG strips of pH 3–10 were purchased from GE HealthCare Biosciences (Immobiline DryStrip, 0.5 mm × 3 mm × 180 mm, Upsala, Sweden). Sodium dodecyl sulfate, acrylamide, methylene-bisacrylamide, TEMED, Tris, glycine, glycerol, and formaldehyde were from Sigma–Aldrich (St. Louis, MO). Dithiothreitol (DTT), urea, CHAPS, Ready Sol Isoelectric focusing (IEF) 40% solutionTM and IPG buffers were also from GE HealthCare Biosciences. Silver nitrate was obtained from Fluka Chemie (Steinheim, Switzerland), and sequencing grade trypsin was from Roche Diagnostics (Mannheim, Germany). Other reagents for 2-DE and MS were purchased from Sigma–Aldrich or Merck (Whitehouse Station, NJ).

CELL CULTURE

HeLa cells were purchased from American Type Culture Collection (Bethesda, MD) and grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (Invitrogen) and 0.05% penicillin–streptomycin (Invitrogen) at 37°C with 5% CO_2 in humidified air.

KNOCK-DOWN OF DEK mRNA AND TRANSIENT TRANSFECTION

pSM2c-DEK expressing DEK specific short hairpin RNAs (nucleotides 654–674 from NM_003472), referred to as shRNA, was purchased from Open biosystems (Hunsville, AL) for knock-down of endogenous DEK expression by RNAi. HeLa cells were seeded at 2.5×10^6 cells/well in 100 mm dish and transient transfection to HeLa cells were performed using Lipofectamine 2000 (Invitrogen) with shDEK or a sh control vector (CTL) for identification of differentially expressed proteins by knock-down of endogenous DEK.

RNA EXTRACTION AND PROTEIN PREPARATION

Total RNA and total proteins were prepared from shDEK transfected cells. For the RT-PCR, total RNA extraction was carried out using TRIZOL reagent (Invitrogen), according to the manufacturer's instructions. Extracted total RNAs were subjected to reverse transcription-PCR. Reverse transcription reactions were performed using M-MLV reverse transcriptase (Promega, Madison, WI) by following the manufacturer's instruction. Total proteins were isolated from shDEK transfected cells using RIPA buffer and which were subjected to Western blot analysis with DEK (Abcam, Cambridge, MA) and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

2-DE

IEF was performed using an IPGphor unit (GE HealthCare Biosciences) with precast non-linear IPG gel strips (18 cm, pH 3–10; GE Healthcare Biosciences). Three hundred micrograms of the total proteins were mixed with rehydration solution (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 M DTT, and a trace of bromophenol

blue) in a final volume of 340 μl and incubated for 12 h at RT before separation by IEF at 500 V for 1 h, 1,000 V for 1 h, or 8,000 V for 5 h (20 mA per gel strip). The gel strips were then immediately equilibrated in equilibrium buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, and 2% (w/v) SDS). Separation in the second dimension was carried out using 10% and 12% SDS–PAGE followed by electrophoresis in a Protean II xi 2D cell (Bio-Rad Laboratories, Hercules, CA) at 10 mA for the first 20 min and then at 20 mA until the bromophenol blue reached the bottom of the gel. The procedure was repeated three times for each sample to ensure reproducibility.

STAINING OF 2D GELS

The 2D gels were stained using a Silver Staining Kit according to the instruction of manufacturer (GE Healthcare Biosciences). Briefly, the gels were fixed in 40% ethanol and 10% acetic acid for 30 min, sensitized in a solution of 25% (w/v) ethanol glutaraldehyde, 5% (w/v) sodium thiosulfate, and 17 g of sodium acetate for 30 min, and washed three times with water for 15 min each. The gels were subsequently immersed in 2.5% (w/v) silver nitrate and 37% (w/v) formaldehyde for 20 min and developed in a mixture of 6.25 g of sodium carbonate and 37% (w/v) formaldehyde for 2–5 min, and the reaction was then stopped in EDTA–Na_2–$2H_2O$.

PROTEOMIC ANALYSIS

Total protein extracts were prepared from untreated, CTL and shDEK samples using a protein extraction solution (1.0 mM PMSF, 1.0 mM EDTA, 1 M pepstatin A, 1 M leupeptin, and 0.1 M aprotinin). 2-DE was performed using an IPGphor IEF unit as described previously [Park et al., 2006] and above. The silver-stained gels were scanned with an ImageScanner (GE Healthcare Biosciences) and analyzed with Phoretix Expression software (ver. 2005; Non-linear Dynamics, Newcastle upon Tyne, UK). Destaining and in-gel tryptic digestion of the protein spots were performed as described [O'Neill et al., 2002]. Xcise (Shimadzu Biotech Co., Kyoto, Japan), an automatic sample preparation system, was used for in-gel digestion, desalting, and plating onto a MALDI-TOF MS plate. Desalting was performed with a ZipTipC18 (Millipore, Eschborn, Germany), and plating was accomplished using a 4-hydroxy-α-cyano-cinnamic acid (HCCA) matrix solution. The in-gel-digested peptides were analyzed using an ultraflex-TOF/TOF (Bruker Daltonics, Bremen, Germany) MALDI-TOF mass spectrometer. The mass spectra were calibrated and processed using Flex Analysis and BioTool 2.2 software (Bruker Daltonics). Peptide mass fingerprinting (PMF) ion searches were performed using Mascot 2.0 software (http://www.matrixscience.-com) integrated with BioTool 2.2. The MSDB and NCBI nr protein databases were searched using the following Mascot settings: taxonomy Mammalia, one incomplete tryptic cleavage allowed, peptide tolerance 50–100 ppm, fragment tolerance 0.5 Da, monoisotopic mass, 1+ peptide charge state as HCCA protonation, alkylation of cysteine by carbamidomethylation as a fixed modification, and oxidation of methionine as a variable modification. For each search, statistically significant ($P < 0.05$) matches were regarded as correct hits. The threshold score for the MSDB was 67, and that for the NCBI database was 65–78.

WESTERN BLOT ANALYSIS

For Western blot analysis, proteins were extracted from three biologically replicated HeLa cells (untreated, CTL, and shDEK transfected cells) and each sample was independently analyzed. Proteins were separated in 12–17% SDS–PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). After blocking with 3% BSA in TBST (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, and 0.05% Tween-20) for over night, and then membranes were detected using the specific antibodies and the protein levels were detected horseradish peroxidase conjugated secondary α-rabbit or α-mouse antibodies. The antibody–antigen complexes detected using the ECL system (Santa Cruz Biotechnology). The primary antibodies used in this study were as followings: α-Prx2, α-PrxVI (AbFrontier, Seoul, Korea), α-prohibitin, α-GRP78, α-annexin V, α-enolase-1, α-bcl-2, α-bax, α-β-actin (Santa Cruz Biotechnology), α-DEK (Abcam), α-caspase-9, and α-caspase-3 antibodies (Cell Signaling Technology, Denvers, MA). The intensity of the chemiluminescence response bands were evaluated using the Quantity One program (Bio-Rad Laboratories).

CHROMATIN IMMUNOPRECIPITATION (ChIP) ANALYSIS

ChIP analysis was performed as described previously [Lee et al., 2008]. Briefly, HeLa cells were transfected with 3 μg of sh control or shDEK vector. Cells were crosslinked with 1% formaldehyde and sonicated for immunoprecipitation with 5 μg of α-acetylated histone H3 or H4 antibodies (Upstate Biotechnology). The immunoprecipitates were eluted and the crosslink reversed, DNA fragments were purified and quantitated using the PCR. The PCR primers designed for Prx VI promoter, intron, and exon analysis were: Prx VI promoter (positions −911 to −672), 5′-GTTGACCTG-CACACAGTAGGTCTC-3′ as a forward primer and 5′-CCTACAGTG-GAGTGGAGTGACTGCT-3′ as a reverse primer; Prx VI final intron (positions 9,353 to 9,708), 5′-GTCATGGCTGTAAAAGTACTGGTG-3′ as a forward primer and 5′-CACTGGAATGGAAGTTCTATGAGGG-3′ as a reverse primer; Prx VI final exon (positions 9,989 to 10,347), 5′-GCTTGGAGAAGAAGCTGCAGAA-3′ as a forward primer and 5′-CTATCCCCATCCTATTGAAAGAC-3′ as a reverse primer.

RESULTS

PROTEOME ANALYSIS TO IDENTIFY CHANGES IN PROTEIN EXPRESSION BY DEK KNOCK-DOWN

Using targeted expression of DEK in Drosophila, it has been reported that DEK induces caspase-dependent apoptosis through its HAT inhibitory activity around anti-apoptotic target gene promoter [Lee et al., 2008]. To further investigate the physiological role mediated by DEK, we applied knock-down system with shDEK and monitored differentially expressed proteins using high resolution 2-DE and MALDI-TOF MS. For proteome analysis, the reduction of endogenous DEK expression after shDEK transfection compare to those of untreated and control vector treated were confirmed by RT-PCR and Western blotting (Fig. 1). Figure 2 shows the 2-DE patterns obtained after separation of 200 μg of total protein from each sample with IEF using IPG strips (pH 3–10, Non-linear). Image analysis of the silver-stained gels allowed us to enumerate the spots as well as determine their levels of expression (vol.%). Of ~2,000 protein spots mapped in

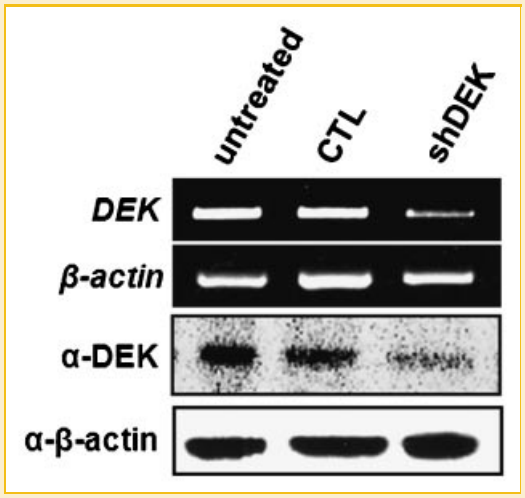

Fig. 1. The reduction of endogenous DEK expression by shDEK plasmid. Knock-down of DEK expression in the shDEK or sh control vector (CTL) transfected HeLa cells was confirmed by RT-PCR and Western blot analysis. β-actin was used as an expression control for equal RNA and protein amounts.

sum on the untreated (Fig. 2A), CTL (Fig. 2B), and shDEK transfected (Fig. 2C) 2-DE gels. Phoretix 2D Expression ver. 2.00 was used to detect variations in the spots between the untreated, CTL, and shDEK. Representative spots from each sample type are shown in Figure 3A,B. Quantification of staining volumes of the individual protein spots from the gels revealed that the vast majority of total protein spots on pH gradient gel were quantitatively similar regardless of whether they represented the control cells and transfected cells with shDEK vector. For the quality and reproducibility of the results, we analyzed three or more gels for each type of cells, and selected those whose normalized volumes were similar for the gels. These 2-DE gels exhibited highly reproducible protein profiles between different cell lines, indicating a low level of experimental variation that could confound data interpretation.

The standardization of 2-DE and increasingly rapid protein identification using mass spectroscopy has made the high-throughput assessment of protein expression changes both practical and cost-effective without prior knowledge of signaling pathways in the cells. Identification of changes in protein expression or protein modification is critical for understanding and dissecting the specific signaling events that regulate various biological processes at the system level. Although detecting changes in signaling proteins, which present at low abundance in cells, is challenging, proteomics offers great potential for studying signal transduction by surveying large number of proteins. Protein spots exhibiting significant changes were excised and subjected to in-gel digestion, followed by identification using MALDI-TOF MS. The quantification of patterns revealed numerous protein spots that seemed to have variable expression in two groups of cells (control and DEK knock-down) judged by their staining intensities. Comparative visual and software-guided analyses of the 2 dimensional protein profiles revealed a consistent change in their expression level on the gels. At

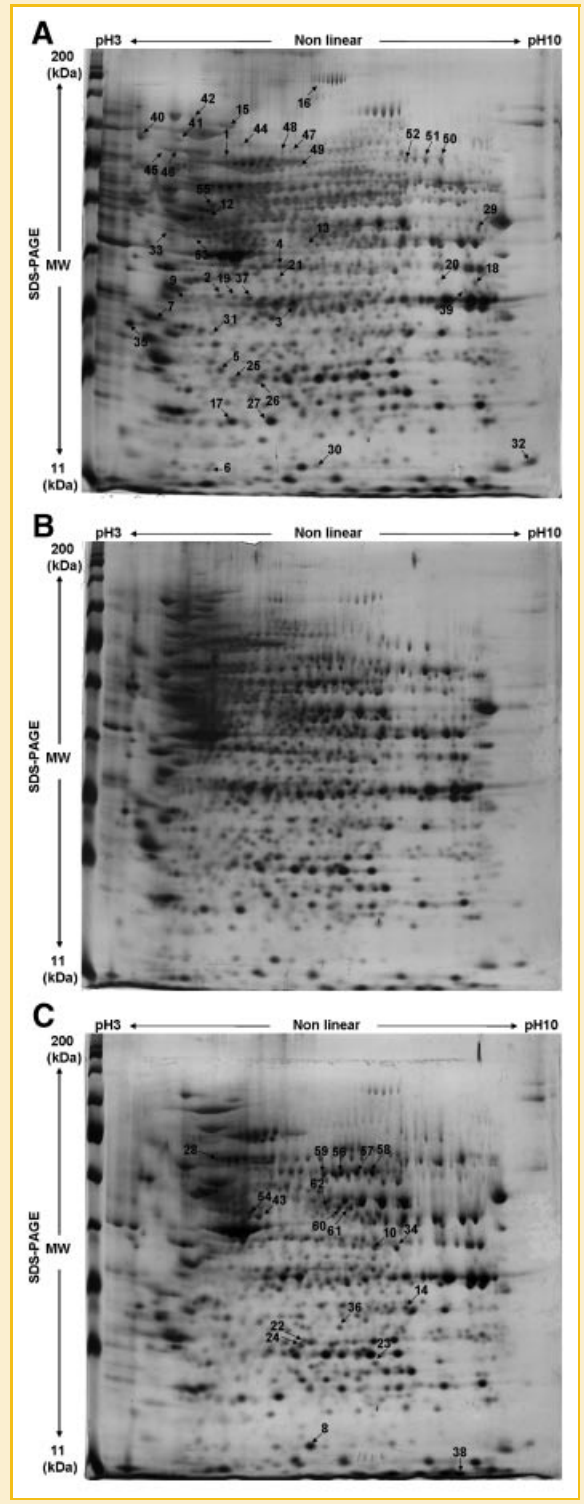

Fig. 2. 2-DE gel electrophoresis. Proteins were isolated from A; untreated, B; control vector (CTL), and C; shDEK. 200 μg of total protein was loaded to the 2-DE gel. First dimension was 18 cm pH 3–10 NL IPG and second dimension was 10% SDS–PAGE gels. They were visualized by the silver staining. Arrows with number show the regulated spots in untreated, CTL, and shDEK transfected samples.

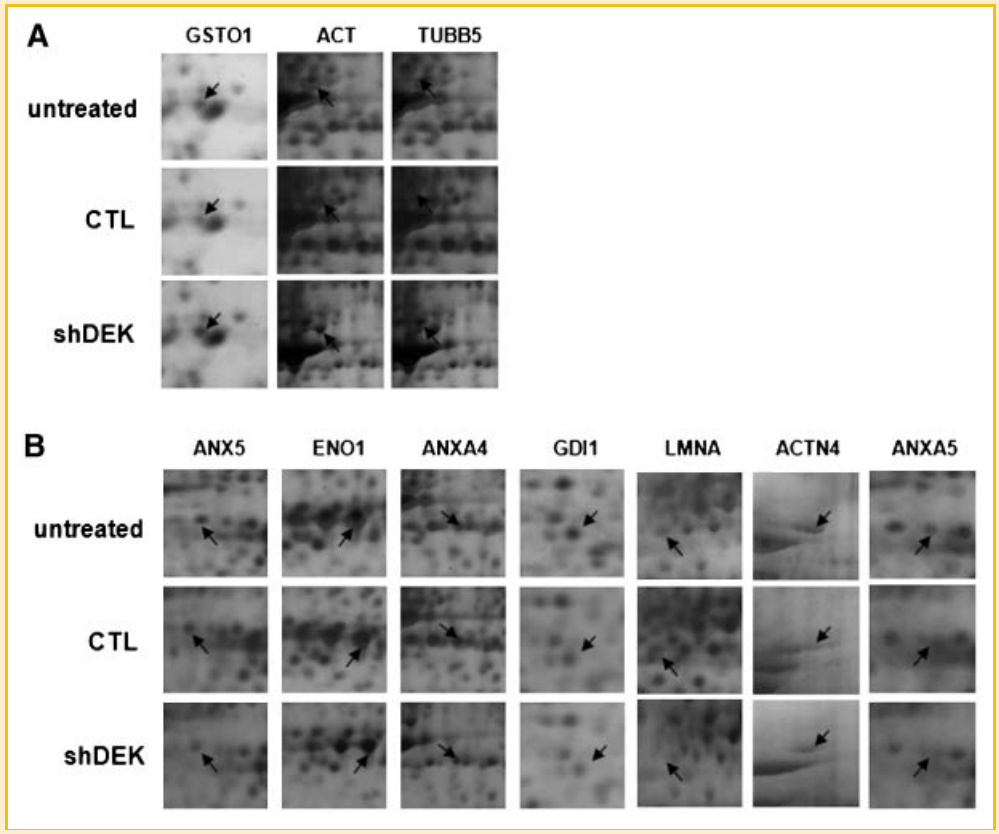

Fig. 3. Examples of differentially expressed spots in untreated, CTL, and shDEK transfected cells. The up-regulated proteins (A) and down-regulated proteins (B) in shDEK transfected cells for knock-down of endogenous DEK. Arrows indicated that differentially expressed protein spots in each sample.

the individual protein level, we analyzed whether separation of the proteins was the best and most reproducible. Then, protein spots exhibiting significant changes in their volumes based on their expression were excised and subjected to in-gel trypsin digestion, leading to the identification of a number of proteins with MALDI-TOF MS and peptide finger printing. For peptide sequencing, the number of analyzed peptides and the Mascot scores are specified. Only Mascot database query results that were statistically significant at the 5% level were further analyzed ($P < 0.05$). MS and protein database analysis of these protein spots identified 58 protein spots. A total of 58 spots were identified as known proteins using the MSDB and NCBI databases. The search results were evaluated on the basis of accepted standards that take into account the number of peptides matched to the candidate protein, the difference in the number of matched peptides between the candidate protein and the next best fit, the coverage of the candidate protein's sequence by matching peptides. Tables I and II summarize the expression change of the up-regulated (1.8- to 3.8-fold changes in expression levels) and down-regulated (1.6- to 5.5-fold changes or reductions to almost undetectable levels) proteins, respectively (Figs. 3 and 4). As shown in these Tables, several spots were shown to be modified forms or isoforms of nearby protein spots, including VCP protein (spot numbers 45 and 46), HSP 70 protein 5 (spots 50 and 51), protein disulfide isomerase-associated three isoform 1 (spots 56, 57, and 58) and heterogeneous nuclear ribonucleoprotein H1 (spots 60 and 61). The identified spots represented 53 unique proteins with the exception of modified forms and isoforms, of which 14 were up-regulated and 39 down-regulated.

CLASSIFICATION OF DIFFERENTIALLY REGULATED PROTEINS BY DEK

A total of 120 spots were shown to be differentially expressed between the untreated, CTL, and shDEK transfected HeLa cells. The representation of identified proteins in several biological process categories differed between the control cells and the DEK knock-down cells. The 58 proteins were assigned to 12 functional categories according to molecular functions and biological process using the information from the Gene Ontology (http://www.geneontology.org) and UniProt (http://www.expasy.uniprot.org) websites (Fig. 5). Figure 3 shows representative features of protein spots, particularly the apoptosis-associated protein spots. The differences in the protein expression levels between the untreated, CTL, and shDEK transfected HeLa cells were further evaluated the amounts of proteins based on the density of the spots analyzed by Phoretix 2D Expression ver. 200 Image software (Fig. 4). Among these, of particular interest was a group of proteins with possible roles in apoptosis, including prohibitin, heat shock 90 kDa protein 1β, actinin α4, HSP 70 protein, glucose-regulated protein (GRP) (78 kDa), peroxiredoxin 2, annexin A5, annexin A4, GDP-dissociation inhibitor (GDI) 1, valosin-containing protein (VCP)

TABLE I. Down-Regulated Proteins in sh-DEK Transfected Cells

Spot no.	Protein name	NCBI accession no.	Swiss Prot accession no.	Method of ID	PMF (MS) Score*	Peptides matched	Peptides obtained	Sequence coverage (%)	Theoretical MW (Da)	Theoretical pI	MS/MS, score
1	BiP	gi|1143492		A	76	15	68	25	72,185	5.03	
2	Annexin δ, lipocortin δ	gi|999926		A	140	14	57	45	35,839	4.98	
3	ENO1 protein	gi|39644728		A	83	10	83	45	29,186	5.87	
4	Annexin A4 (annexin χ)	gi|4033507		A	106	11	41	39	36,034	5.71	
5	GDP-dissociation inhibitor 1, chain B	gi|7245833	1DOAB	A	100	16	56	54	23,005	5.2	
6	Peroxiredoxin 2	gi|1717797	P52552	A	63	7	83	45	14,272	4.7	
7	40S ribosomal protein SA(p40)(34/67 kDa laminin receptor)	gi|730679	P38982	A	70	7	33	30	32,881	4.7	
9	Laminin receptor 1	gi|74750453	Q86VC0	A	66	6	34	30	32,977	4.6	
12	Thioredoxin domain-containing 2	gi|29477099		A	85	10	40	25	53,637	4.92	
13	Lamin A isoform A	gi|220474		A	73	10	41	26	47,792	6.63	
15	Actinin, alpha 4, isoform CRA_c	gi|119577215		A	79	12	35	15	104,555	5.24	
16	PREDICTED: Similar to ciliary rootlet coiled-coil, rootletin	gi|113414344		A	67	15	59	10	190,849	6.32	
17	Immunoglobulin heavy chain variable region	gi|47846749		A	69	6	82	62	12,343	5.59	
18	Chain A, human annexin A2 with heparin tetrasaccharide bound	gi|114794644		A	77	8	43	26	35,448	8.21	
19	Annexin A5	gi|113960	P08758	A	65	8	45	33	35,971	4.94	
20	Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1, isoform CRA_d	gi|119574080		A	83	10	84	38	30,942	7.03	
21	Cofilin 1 (non-muscle)	gi|5031635	P23528	A, B	66	8	81	58	38,262	6.25	35,49
25	p64 CLCP	gi|895845		A, B	104	11	86	62	23,813	5.12	22,39
26	Chain A, crystal structure of Hgstp1-1[v104] complexed with the Gsh conjugate of (+)-anti-Bpde	gi|5822569		A, B	95	11	87	56	23,506	5.43	48,70,49
27	Cellular retinoic acid binding protein 1	gi|4758052	P29762	A, B	107	11	95	71	15,727	5.3	67
29	Phosphoglycerate kinase 1	gi|52788229	P00558	A, B	68	13	77	42	44,985	8.3	48
30	Histidine triad nucleotide-binding protein 1	gi|1708543	P49773	A, B	67	55	65	14	6.43		87
31	Microtubule-associated protein RP/EB family member 1	gi|20138589	Q15691	A, B	65	71	35	30	5.02	26	94
32	Neuropolypeptide h3	gi|4261934	P30086	A, B	66	7	84	57	16,068	8.81	37,73
33	Calreticulin precursor	gi|62897681		A, B	74	11	76	31	47,061	4.3	19,21
35	Ribosomal protein SA	gi|9845502	P08865	A	111	11	70	47	32,947	4.79	
37	Prohibitin	gi|46360168	P35232	A, B	69	9	83	48	29,859	5.57	37
40	Glucosidase II	gi|2274968	Q14697	A	134	27	99	29	107,289	5.71	
41	Glucosidase, alpha; neutral AB, isoform CRA_a	gi|119594451		A	103	16	40	17	104,930	5.85	
42	Neutral alpha-glucosidase AB [precursor]	gi|54037162	Q14697	A	123	22	75	30	107,263	5.74	
44	Heat shock protein gp96 precursor	gi|15010550	P14625	A	73	8	29	14	90,309	4.73	
45	VCP protein	gi|48257098	Q96IF9	A	125	22	94	40	71,534	4.94	
46	VCP protein	gi|48257098	Q96IF9	A	75	13	60	30	71,534	4,394	
47	Leucine praline-enriched proteoglycan (leprecan) 1, isoform CRA_d	gi|119627550		A	71	13	67	22	84,178	5,005	
48	Heat shock 90 kDa protein 1, beta	gi|20149594		A	85	13	60	24	83,554	4.97	
49	Ezrin	gi|46249758	Q6NUR7	A	68	13	53	23	69,313	5.94	
50	GRP78 precursor, 78 kDa glucose-regulated protein, heat shock 70 kDa protein 5	gi|386758	P11021	A	184	26	93	41	72,185	5.03	
51	GRP78 precursor, 78 kDa glucose-regulated protein, heat shock 70 kDa protein 5	gi|386758	P11021	A	145	19	52	32	72,185	5.03	
52	Heat shock cognate 71 kDa protein	gi|123648	P11142	A	86	14	49	28	71,082	5.37	
53	Vimentin	gi|62414289	RUVB1	A	173	21	45	46	53,676	5.06	
55	Mitochondrial ATP synthase, H+ transporting F1 complex beta subunit	gi|89574029	Q0QEN7	A	88	15	65	39	48,083	4.95	

A, MALDI-TOF.
B, MALDI-TOF/TOF MS.
*Score thresholds were 65–78 for NCBI database.

and cofilin 1 (non-muscle). These apoptosis-associated proteins accounted for 16% of the down-regulated proteins in the shDEK transfected HeLa cells. Another major group of proteins included the regulation of programmed cell death and regulation of apoptosis proteins, which comprised 15% of the down-regulated proteins in the shDEK transfected HeLa cells. In addition, under the molecular function heading are proteins predicted to have oxidoreductase activity (9%), antioxidant activity (3%), and thioredoxin peroxidase activity (2%). Interestingly, more proteins with oxidoreductase activity were significantly up-regulated in shDEK transfected HeLa cells. Based on these the differential expression patterns on 2-DE gels due to the presence of DEK, we provide some evidence that these apoptosis-related proteins may represent the role of DEK in the biological process, although the molecular basis by which these proteins identified in this study control these cellular responses is still to be elusive.

VALIDATION OF PROTEOMIC RESULTS BY WESTERN BLOT ANALYSIS

We tried to confirm the differential expression of the identified protein spots with control and DEK knock-down cells and analyzed individual changes using Western blot analysis. In those cases where

TABLE II. Up-Regulated Proteins in sh-DEK Transfected Cells

Spot no.	Protein name	NCBI accession no.	SwissProt accession no.	Method of ID	PMF (MS) Score*	Peptides matched	Peptides obtained	Sequence coverage (%)	Theoretical MW (Da)	Theoretical pI	MS/MS, score
8	GSTM4	gi|68521878		A	104	8	41	38	18,672	5.93	
10	Peroxiredoxin 4	gi|16758274		A	82	12	58	37	31,216	6.18	
14	Glutathione transferase omega 1	gi|20141290	Q9N1F5	A	65	9	35	35	27,743	6.84	
23	Thiol-specific antioxidant protein	gi|438069	P32119	A, B	83	9	100	44	22,014	6.84	33,29
24	Peroxiredoxin 6	gi|4758638	P30041		132	14	71	62	25,133	6	43
28	Protein disulfide-isomerase [precursor]	gi|20070125	P07237	A	79	14	100	38	57,480	4.76	
34	Aldolase A protein	gi|28595		A, B	74	7	93	74	39,851	8.3	37
36	Phosphoglycerate mutase 1	gi|4505753	Q53G35	A, B	125	12	95	44	28,900	6.67	40,59
38	Chain A, cyclophilin A complexed with dipeptide Gly-Pro	gi|1633054		A, B	73	9	82	54	18,098	7.82	35
43	Actin, cytoplasmic 1	gi|46397333		A	62	7	24	23	42,052	5.29	
54	Tubulin, beta 5	gi|7106439		A	168	24	86	54	50,095	4.78	
56	PREDICTED: Protein disulfide isomerase-associated 3 isoform 1	gi|114656687		A	129	22	69	41	55,328	6.42	
57	PREDICTED: Protein disulfide isomerase-associated 3 isoform 1	gi|114656687		A	129	18	46	32	55,328	6.42	
58	PREDICTED: Protein disulfide isomerase-associated 3 isoform 1	gi|114656687		A	114	18	69	37	55,328	6.42	
59	Chain A, human mitochondrial aldehyde dehydrogenase complexed with Nad+ and Mn2+	gi|6137677		A	92	12	56	29	54,394	5.70	
60	Heterogeneous nuclear ribonucleoprotein H1	gi|5031753	P31943	A, B	88	8	37	28	49,484	5.89	28
61	Heterogeneous nuclear ribonucleoprotein H1	gi|5031753	P31943	A	80	12	57	36	49,484	5.89	

A, MALDI-TOF.
B, MALDI-TOF/TOF MS.
*Score thresholds were 65–78 for NCBI database.

the antibodies were commercially available for western blot analysis, we tested their differential expression with protein extracts from three different kinds of HeLa cells for the verification. Protein extracts from untreated, CTL, and shDEK treated cells were blotted onto membrane and further incubated with specific antibodies against the proteins identified by 2-DE. Changes in the expression level of proteins tested were fully consistent with the 2-DE images (Fig. 6). The levels of annexin V (ANX5), GRP78, prohibitin (PHB), peroxiredoxin 2 (Prx 2), and VCP were significantly decreased in shDEK cells and peroxiredoxin VI (PrxVI) protein was relatively increased when DEK is knock-down. Thus, our 2-DE based proteomic and immunological tools provided important clues for the identification of differentially regulated and functionally related proteins by proto-oncogene DEK and opened new clues for the study of signaling dissection generated by this proto-oncogene.

TRANSCRIPTIONAL REGULATION OF PEROXIREDOXIN VI PROMOTER VIA HISTONE ACETYLATION

We next investigated whether DEK is involved in histone modifications on regulated proteins when DEK was knocked down. Among the regulated proteins in DEK knock-down cells, we focused the Prx VI, which was up-regulated in our proteome analysis. Since HAT inhibitory activity of DEK was decreased in DEK knock-down cells, we reasoned that the promoter region would be hyperacetylated and that will lead to transcriptional activation of Prx VI gene. We performed ChIP analysis with anti-acetylated histone H3 and H4 antibodies, and compared the acetylation levels in promoter, intron, and exon of Prx VI (Fig. 7A). To use the constant DNA concentrations, input was used as a positive control and mouse IgG was used for negative control from HeLa cells transfected with either sh control and shDEK vector. As expected, we found that knock-down of DEK protein resulted in both H3 and H4 hyperacetylation status of Prx VI promoter region (Fig. 7B). On the contrary, other two regions, intron and exon did not show any changes in histone H3 and H4 acetylation levels (Fig. 7C,D). These results suggest that DEK protein have important role in transcriptional regulation of Prx VI via its HAT inhibitory activity.

CHARACTERIZATION OF THE DEK KNOCK-DOWN CELLS BASED UPON THE APOPTOTIC RELATED PROTEINS

To further correlate the proteome profiles to the physiological function of DEK related to the apoptotic processes in the cells, other important regulators in apoptosis pathway were measured by Western blot analysis in control, CTL and shDEK treated cells. Consistent with our previous studies, downstream regulators, caspase-9 and -3 were down-regulated when DEK is knocked down by showing increased procaspase-9 (p46) and decreased active form of caspase-3 (Fig. 8). In addition, expressions of a proapoptotic protein, Bax, and of an anti-apoptotic protein, Bcl-2, reciprocally depend on the levels of expression of DEK (Fig. 8). DEK knock-down led to a reduction of Bax levels, whereas DEK down-regulation resulted in elevated expression of Bcl-2. This is likely to reflect direct and indirect influences of DEK on functions of anti-apoptotic proteins (such as Bcl-2, Bcl-X_L, Mcl-1) and proapoptotic proteins (such as Bax, Bak, Bad, Bik, Bid, Bok). However, profiling of proteins and identification of the in vivo targets of DEK upon DEK overexpression and knock-down will be needed to more precisely elucidate the molecular mechanisms by which DEK renders cells resistant to apoptosis.

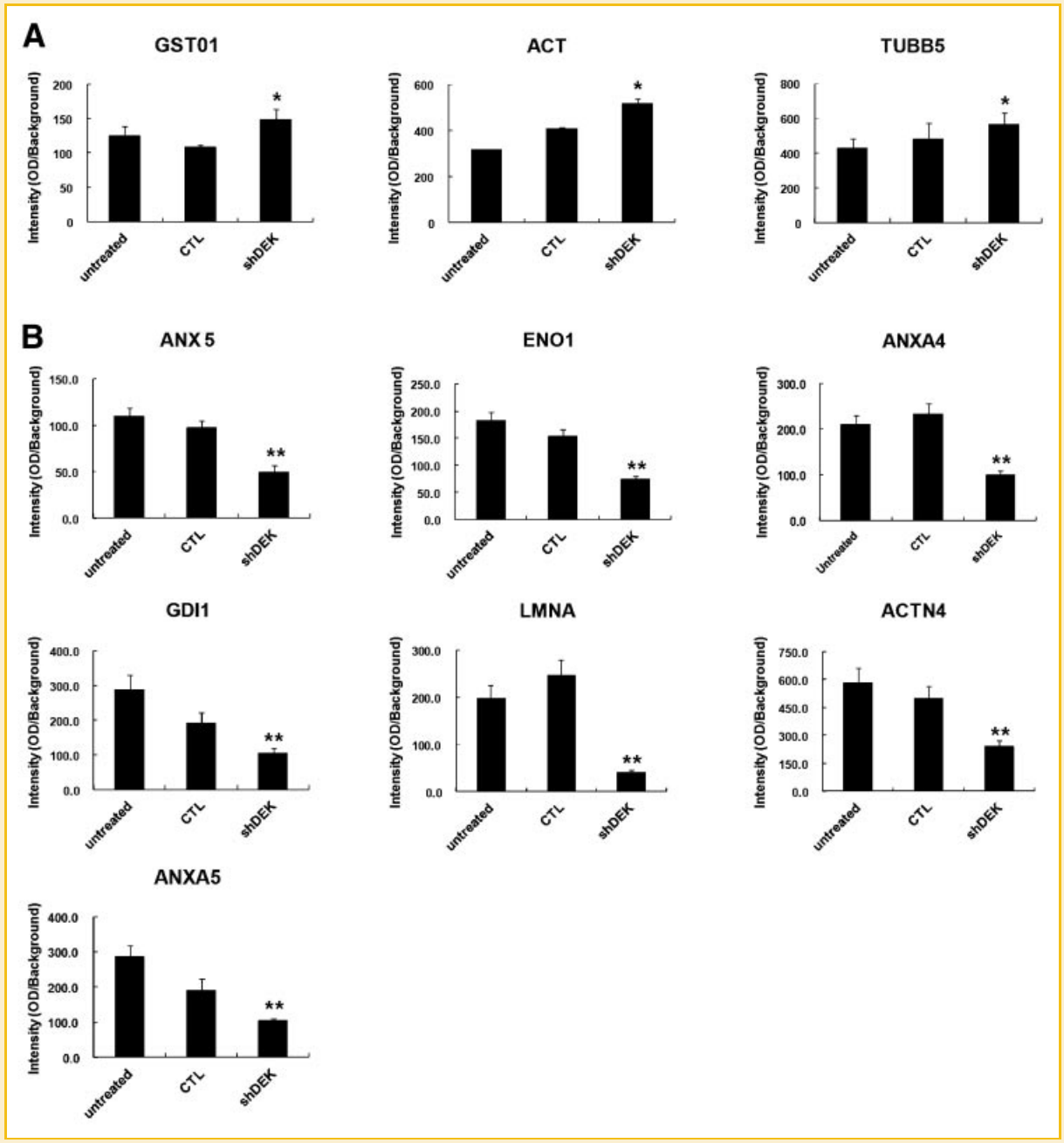

Fig. 4. Regulated proteins in DEK knock-down HeLa cells by shDEK. Each graph shows that relative volume intensities of regulated proteins, which were calculated and graphically presented by Phoretix Expression software. The up-regulated (A) and down-regulated proteins (B) in shDEK transfected cells. The intensity volume (OD/Background) of each individual spot (relative to the intensity volumes of all spots) was used for the comparative analysis with unpaired Student's *t*-test. *P*-values <0.05 were considered statistically significant. For all panels, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with CTL. ACT, actin cytoplasmic 1; TUBB5, tubulin beta 5; ANXA4, annexin A4; ANXA5, annexin A5; GDI1, GDP-dissociation inhibitor 1 chain B; LMNA, lamin A; ACTN4, actinin alpha 4.

DISCUSSION

Herein we present our systematic approaches to dissecting proteome changes due to proto-oncogene DEK in HeLa human cervical adenocarcinomas. We used 2-DE in combination with image analysis for protein expression changes and identification with MALDI-TOF MS to study changes in the global expression pattern by taking its advantages of easy quantification. Although there is a lack of detailed knowledge regarding the signaling connection with physiology of DEK expression, it should be noted that data represent the most direct quantitative evidence that these changes take place at the protein levels and these differentially expressed proteins seen

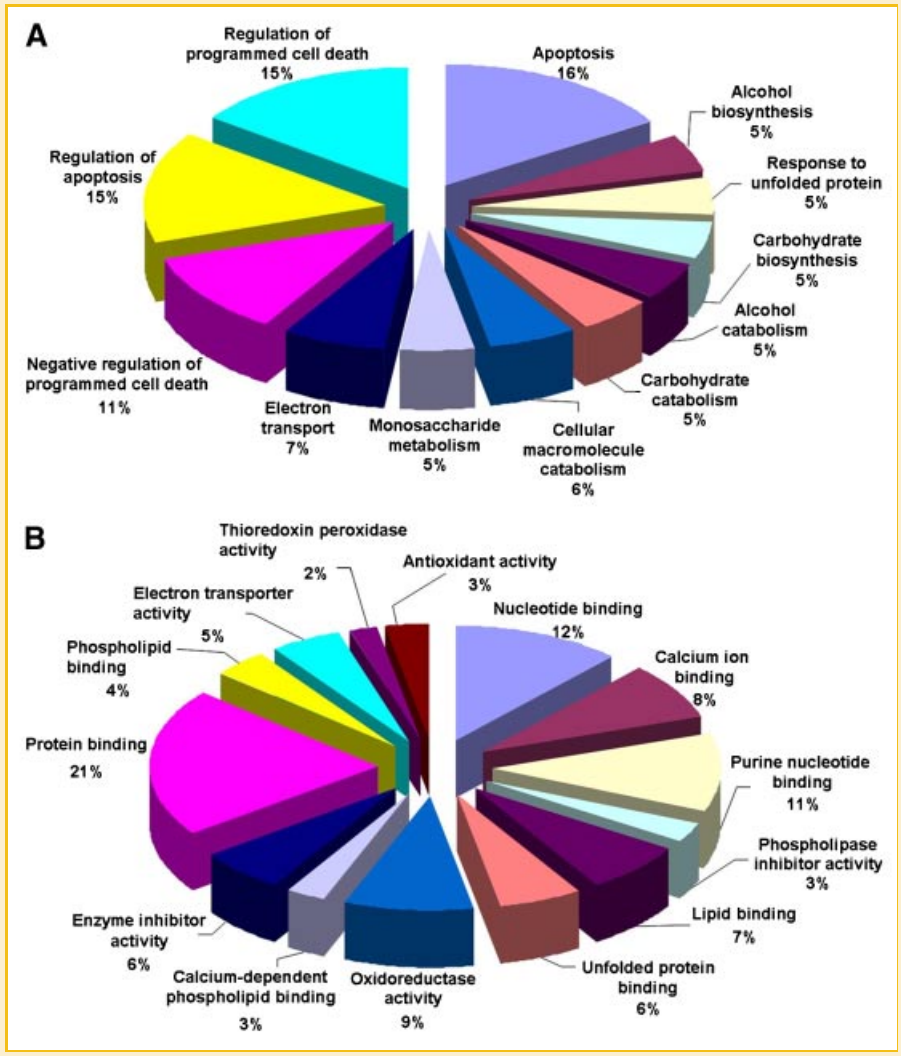

Fig. 5. Classification of differentially regulated proteins from the proteomic analysis. Ontological classification of differentially regulated proteins in terms of (A) Biological Process, (B) Molecular Function using the Gene Ontology (http://www.geneontology.org) and UniProt (http://www.expasy.uniprot.org) websites. The compositions of identified proteins are presented as percentages of all the individual identified proteins. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

here may be targets for the function and physiology of the proto-oncogene DEK. The interpretation of these findings is couched in current knowledge regarding the function of this protein in the cells. Thus, this proteome profiling provides the windows for understanding the proteins involved in functional varieties that might contribute to DEK-controlled cellular phenotypes in an in vitro model system as a mimic of naturally occurring cells in vivo, especially relationship to apoptosis. Regulation of apoptotic cell death is a complex process affected by a number of proteins and involves many signaling pathways. Apoptosis is indeed one of the main pathways involved in tumor progression as well as senescence [Collado et al., 2007]. In the present study, we report our findings on the application of the proteomic approach to the characterization of proteins differentially expressed by DEK knock-down, which DEK proto-oncogene protein is a nucleic acid binding protein involved in carcinogenesis, autoimmune disease, and viral infection. It has been reported that cell death in response to DEK depletion was accompanied by increased protein stability and transcriptional activity of the p53 tumor suppressor and consequent up-regulation of well known p53 target genes such as $p21^{CIP}$ and [Ye, 2005; Wise-Draper et al., 2006]. Of particular interest was that the large part of the differentially regulated proteins due to DEK depletion with shDEK transfection was apoptosis-related protein networks. These findings were consistent with the previous notion for DEK mediated apoptosis.

The protein identifications in our 2-DE-based proteomics screening suggested that multiple cellular processes were involved in function and physiology of proto-oncogene DEK protein. The identified proteins are involved in a range of molecular functions and biological processes, indicating that a wide representation of proteins was identified. The representation of identified proteins in several biological process categories into 12 functional categories,

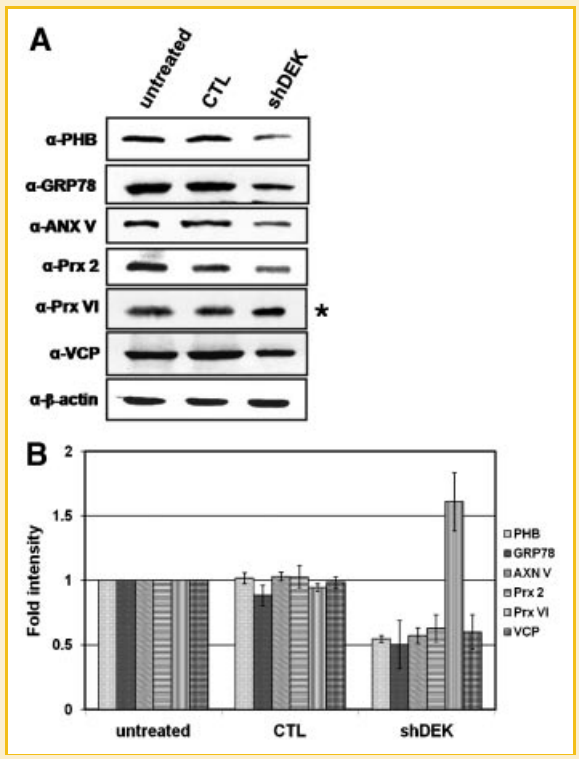

Fig. 6. Western blot analysis of regulated proteins in 2-DE results by shDEK transfection. A: Total proteins were extracts from shDEK transfected HeLa cells and then which were subjected to Western blot analysis using specific antibodies recognizing identified proteins in 2-DE. β-actin was used as a loading control. Asterisk indicates that up-regulated protein in shDEK transfected cells and abbreviation of proteins is explained in the text. B: Western blot images were analyzed quantitatively in bar graph by Quantity One program. The data re averages of three independent experiments, and the error bars represent ±standard deviation.

including regulation of apoptosis, electron transport, carbohydrate metabolism, and catabolism, etc. Among the down-regulated proteins, annexins have many functions through interaction with cell membrane components that are involved in several physiological processes, such as structural organization, intracellular signaling, and growth control in cell [Moss and Morgan, 2004]. In many carcinomas, annexins were differentially expressed in their endogenous protein levels. For example, annexin A5 and A1 were down-regulated in leukemias [Gerke and Moss, 2002] and prostate cancers [Emmert-Buck et al., 2000]. Also, expression level of annexin A4 was significantly decreased in hormone refractory prostate cancer [Xin et al., 2003]. These changes in the levels of expression of annexins might be effects the motility, invasiveness, and proliferative rate of cell [Gerke and Moss, 2002]. Enolase is the glycolytic enzyme that catalyzes the formation of phosphoenolpyruvate from 2-phosphoglycerate. Enolase-1 (ENO1) cDNA have 97% similarity with MBP-1 (Myc-binding protein-1). ENO1 and MBP-1 strongly suggest that were encoded by the same gene. Furthermore, recent findings have shown that MBP-1 was involved in regulation of cell growth and apoptosis [Ray et al., 1995; Ghosh et al., 2002, 2005]. These reports suggest that overexpression of ENO1 might be induces apoptosis in cells through activation of mitochondrial apoptosis pathway. Lamins are a nuclear membrane protein involved in cell cycle control, chromatin organization, cell differentiation, and apoptosis [Gruenbaum et al., 2000]. Recent report shows that most tumors show a down regulation of lamin A expression, such as small cell lung carcinomas [Broers and Ramaekers, 1994], testicular cancer [Machiels et al., 1997]. Furthermore, absence of lamin A expression was correlated with rapid growth in basal cell carcinomas [Venables et al., 2001]. Recently, increased level of Glutathione-*S*-transferase omega 1 (GSTO1) was detected in papilloma induced murine skin, which

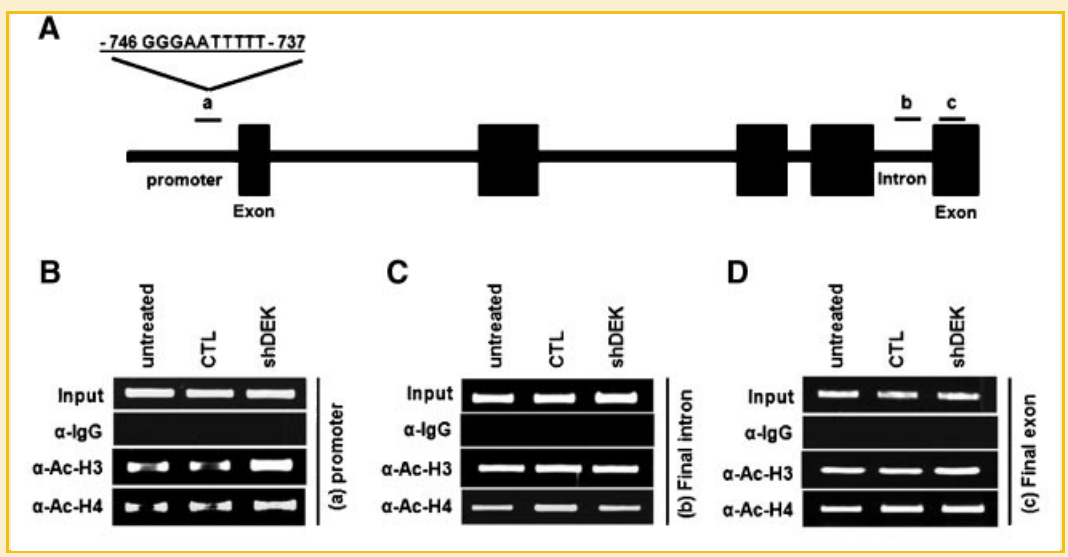

Fig. 7. Analysis of histone acetylation status on promoter of up-regulated protein Prx VI. A: Genomic structure of Prx VI gene and above lines indicates the amplified fragments by PCR. B–D: ChIP analysis on promoter, intron, and exon of Prx VI. The transfected HeLa cell lysates with sh control or shDEK vector were ChIP analysis using mouse IgG, anti-acetyl H3 and H4 antibodies. The immunoprecipitated DNA was amplified by PCR from promoter (a), final intron (b), and final exon (c).

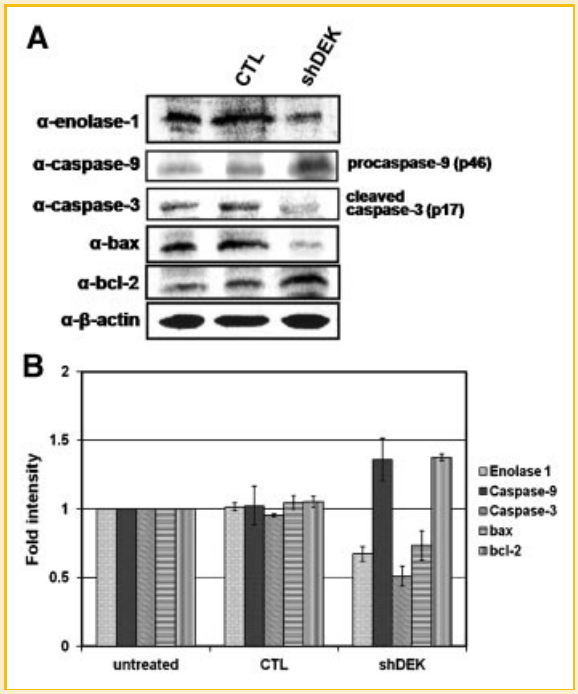

Fig. 8. Analysis of apoptosis related proteins in shDEK transfected cells. A: Cell extracts were prepared from HeLa cells which induced knock-down of endogenous DEK. Expression of apoptosis related proteins were detected by Western blot using specific antibodies. β-actin was used as a loading control. B: Western blot images were analyzed quantitatively in bar graph by Quantity One program. The data are averages of three independent experiments, and the error bars represent ±standard deviation.

expression showed in proliferative areas of the papillomas [Ridd et al., 2006]. These results provide that change of GSTO1 expression level is associated with tumor formation through high expression within the initiated cells prior to papilloma formation. In our results, we realized that Prx VI was up-regulated in shDEK cells. Recent report suggests that as a cellular antioxidant enzyme and a member of the peroxiredoxin superfamily, Prx VI increases apoptotic cell death (~80% at 48 h) in Prx VI down-regulated L2 cells by specific antisense oligonucleotide treatment [Pak et al., 2002]. Using ChIP analysis, we have demonstrated that knock-down of DEK which has HAT inhibitory activity caused hyperacetylation of histones around Prx VI promoter and probably up-regulated its expression.

Previously, we have shown that DEK can induce caspase-3 and -9 dependent apoptosis when overexpressed in Drosophila model system [Lee et al., 2008]. Furthermore, we found that chromatin hypoacetylation of bcl-2 promoter and therefore repression of anti-apoptotic protein bcl-2 contributes DEK mediated apoptosis. We confirmed that ENO1, which is involved in apoptotic pathway along with MBP1 is down-regulated when DEK is knocked down. Other important regulators in apoptosis pathway were measured by Western blotting in shDEK treated cells. Consistent with our previous studies, downstream regulators, caspase-9 and -3 were down-regulated when DEK is knocked down by showing increased procaspase-9 (p46) and decreased active form of caspase-3 (p17) (Fig. 8). It is interesting that bax is down-regulated which might suggest that DEK mediated signal induces ENO1 expression and then the signal activates bax to migrate from cytoplasm to mitochondria membrane resulted in cytochrome *c* release. It is intriguing that PARP which is activated by the presence of DNA breaks and an inducer of apoptosis is down-regulated in shDEK treated cells. Recent report suggests that DEK is released to extracellular space upon poly (ADP-ribosylation) and then caspase activation might be followed [Kappes et al., 2008]. Consistent with our previous study, antiapoptotic bcl-2 which blocks cytochrome *c* release from mitochondria is decreased in shDEK cells.

In conclusion, we have demonstrated the successful application of a simple proteomic approach to profiling the proteome changes by using a 2-DE/MALDI-TOF-MS based technology that specific proteins involved in apoptosis pathway are differentially expressed by DEK knock down. Construction of biological networks related to proteome profile due to proto-oncogene DEK knock-down revealed many known and unexpected proteins and, of interest, apoptotic pathway that play keystone roles in function and physiology of proto-oncogene DEK. These observations provide some insights into the function and physiology of DEK at the proteome level. Although we have demonstrated the list of apoptosis related proteins with increased or decrease expression upon DEK knock down in this study, further research should be followed to elucidate the specific target proteins of DEK in its role in apoptosis pathway and its regulation.

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