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The Human Homolog of Diminuto/Dwarf1 Gene (hDiminuto): A Novel ACTH-Responsive Gene Overexpressed in Benign Cortisol-Producing Adrenocortical Adenomas

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To elucidate the molecular mechanism of the pathogenesis of benign functioning adrenocortical adenomas causing Cushing's syndrome, we employed suppression PCR-based cDNA subtractive hybridization to identify novel genes that are differentially expressed in the adenoma. In this report we describe the adenoma-specific overexpression of the human homolog of the Diminuto/Dwarf1 (hDiminuto) gene. Northern blot analysis revealed that hDiminuto mRNA was overexpressed in the adenoma tissue of 14 patients with Cushing's syndrome in comparison to the adjacent nontumorous adrenal gland. *In situ* hybridization using hDiminuto cRNA probe showed its abundant expression in the tumor cells, whereas the nontumorous cells showed a low level of expression. As the atrophic adjacent gland may not represent the normal architecture, we examined the expression pattern of hDiminuto mRNA in normal human adrenal cortex. *In situ* hybridization revealed that it was expressed in all layers of the normal adrenal cortex. *In situ* apoptosis detection by the TUNEL method revealed that a low level of hDiminuto expression in the atrophic, adjacent gland was associated with numerous TUNEL-positive cells in all layers of cortex. In contrast almost no apoptotic cell was detected in the tumor or in the normal adrenal cortex where hDiminuto expression was abundant. These results are compatible with a recent report that hDiminuto acts as an antiapoptotic factor in neurons. The expression of hDiminuto in the normal adrenal cortex was most abundant in the zona fasciculata, suggesting its possible regulation by ACTH/cAMP. Indeed, forskolin treatment of H295R human adrenocortical cells resulted in a significant induction of the mRNA in a time- and dose-dependent manner. To further demonstrate the physiological regulation, an *in vivo* experiment was carried out in dexamethasone-treated rats. ACTH administration to these rats increased the mRNA expression. These results led us to speculate that the overexpression of hDiminuto in the adenoma could be due to the abundant expression of ACTH receptor, as we previously described. Diminuto is involved in steroid synthesis and cell elongation in plants. We, therefore, hypothesize that hDiminuto might be involved in the molecular events of adrenocortical tumorigenesis by facilitating steroid synthesis and cell growth. (*J Clin Endocrinol Metab* 86: 5130–5137, 2001)

AN EPIDEMIOLOGICAL STUDY in Japan from 1987–1992 revealed that benign cortisol-producing adrenocortical adenomas account for 40% of the cases of Cushing's syndrome (CS) (1). Although it is a major cause of CS, the molecular events in the pathogenesis of these adenomas are still not well characterized. The hallmarks of adrenocortical adenomas are increased cell proliferation and steroidogenesis. Both of these events are positively regulated by ACTH (2). However, neither an activating mutation in ACTH receptor (ACTHR) nor abnormalities in the post-ACTH signal transduction pathway have been identified in these adenomas to date (3–5). Recently, we reported the overexpression of the ACTHR gene in these adenomas and speculated that increased expression of ACTHR, in the absence of circulating ACTH, might activate the intracellular signal transduction pathway to augment steroidogenesis (3). Mutations in tumor suppressor gene p53, overexpression of IGF-II, and high inhibin-like immunoreactivity have been reported in the adenoma (6–9). However, these findings could not be demonstrated by separate studies, and the relevance of these findings to tumorigenesis has not been corroborated (10). In view of this background we endeavored to identify genes that are selectively up-regulated in benign cortisol-producing adenomas and that might have some role in the process of tumorigenesis.

We employed suppression PCR-based cDNA subtractive hybridization to isolate genes differentially up-regulated in the adenoma in comparison to the atrophied nontumorous adrenal gland (11, 12). This procedure allows the isolation of differentially expressed cDNAs among two different cDNA populations, called Tester and Driver. Usually, the Driver cDNA population is subtracted from the Tester cDNA population by hybridization. Thus, cDNAs present only in Tester are enriched and amplified by PCR. In our study we employed cDNA derived from the adenoma tissue as the Tester and that from the adjacent atrophied nontumorous gland as the Driver. We cloned several cDNAs that are selectively

Abbreviations: ACTHR, ACTH receptor; CS, Cushing's syndrome; FAD, flavin adenine dinucleotide; FITC, fluorescein isothiocyanate; GAPDH, glyceryldehyde-3-phosphate dehydrogenase; hDiminuto, human homolog of Diminuto/Dwarf1; ISH, *in situ* hybridization; 5′-RACE, rapid amplification of 5′-cDNA ends; rDiminuto, rat Diminuto.

up-regulated in the adenoma. As expected, sequence analysis revealed that most of the steroidogenic enzymes and steroidogenic acute regulatory protein mRNAs were overexpressed in the adenoma. In addition, several mitochondrial genome-encoded RNAs, such as cytochrome oxidase III, NADH dehydrogenase, and adenosine triphosphatase, were also found to be up-regulated in the adenoma. These genes are up-regulated by ACTH in adrenocortical cells and are suggested to meet the metabolic needs of steroid hormone production by enhancing the energy-producing capacity of steroidogenic cells (13). However, overexpression of no known oncogene could be detected in the adenoma. We, therefore, looked for genes that are involved in cell proliferation and steroid synthesis and thus might be involved in the molecular events of adrenocortical tumorigenesis. One of them was glutathione-*S*-transferase A1, as we reported previously (14). Sequence analysis revealed that another clone encodes the human homolog of Diminuto/Dwarf1 (hDiminuto; GenBank accession no. D13643).

The function of hDiminuto in adrenocortical cells is not known. However, in *Arabidopsis thaliana* the function of Diminuto has been well characterized. Diminuto is involved in regulating cell elongation and steroid synthesis in this species (15, 16). The *dim* mutant, harboring a mutation in the diminuto gene, is a severe dwarf with greatly reduced fertility. Diminuto is required for brassinosteroid synthesis, which is an essential plant sterol necessary for longitudinal growth and the development of plant cells. Recently, hDiminuto has been cloned from human brain by Greeve *et al.* (17). The researchers termed it seladin-1 (selective Alzheimer's disease indicator 1) and showed that it serves as an antiapoptotic factor. In the present study we analyzed the expression profile of hDiminuto mRNA in adenoma and normal human adrenal gland. We also studied the regulation of its expression and speculated its function in adrenocortical cells.

Subjects and Methods

Case subjects

Fourteen patients with Cushing's syndrome were studied. Written informed consent was obtained from all patients for the use of their surgical specimens. The clinical data of the patients were presented in our previous study (3). The patients were treated by unilateral adrenalectomy.

Sample collection

After adrenalectomy, a portion of resected adenoma and adjacent atrophic adrenal tissue were immediately frozen in liquid nitrogen and kept at −80 C. The rest of the tissue was used to make formalin-fixed, paraffin-embedded sections.

Cell culture

The human adrenocortical cell line H295R (American Type Culture Collection, Manassas, VA; CRL-2128) was cultured as described previously (14). The cells were treated with forskolin (Wako, Tokyo, Japan) at different time intervals and concentrations.

Animal treatment

Male Wistar rats, weighing approximately 180 g, were maintained under controlled temperature (23 ± 1 C) and lighting (12-h light/12-h dark cycle) with access to food and water *ad libitum*. Each group consisted of four rats. To all rats except the control group, dexamethasone (Decadron, Banyu, Tokyo, Japan) was administered ip once a day at a dose of 4 mg/kg BW for 3 d. After dexamethasone treatment, ACTH (Cortrosyn, Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan) was injected ip once a day at a dose of 50 IU/kg BW. The rats were killed by decapitation at 12, 24, and 48 h after ACTH injection. The adrenal glands were excised, cleaned of adrenal fat, weighed, frozen immediately in liquid nitrogen, and stored at −80 C until total RNA extraction.

Extraction of RNA and cDNA synthesis

Total RNA from the adrenal glands of the patients was extracted using the QIAGEN RNA/DNA kit according to the manufacturer's protocol (QIAGEN, Hilden, Germany). Total RNA from six patients (170 ng each) were pooled for the adenoma and the adjacent atrophic tissue. Thus, 1 μg total RNA each from the adenoma and the adjacent atrophic tissue was used to prepare double stranded cDNA with the SMART PCR cDNA synthesis kit (CLONTECH Laboratories, Inc., Palo Alto, CA) according to the manufacturer's protocol. Total RNA from rat adrenals was extracted according to the method of Chomczynski and Sacchi (18).

Suppression PCR-based cDNA subtractive hybridization

Double stranded cDNA was used to perform suppression PCR-based cDNA subtractive hybridization using the PCR-select cDNA subtraction kit (CLONTECH Laboratories, Inc.) as described previously (14). The cDNAs from the adenoma and the adjacent atrophic tissue were regarded as Tester and Driver, respectively. The amplified products were cloned into pGEM-T Easy vector (Promega Corp., Madison, WI), which was used to transform JM109 *Escherichia coli*. The screening of the clones differentially expressed in the adenoma was carried out as described previously (14).

Rapid amplification of 5′-cDNA ends (5′-RACE)

5′-RACE was carried out using the 5′-RACE System for Rapid Amplification of cDNA Ends kit (Life Technologies, Inc.) according to the manufacturer's protocol. Total RNA obtained from human adrenal gland was used to perform 5′-RACE of hDiminuto cDNA. The primers used were: gene specific primer 1, 5′-CAC CCA CAG CAG GAA GAG CAG-3′; and gene specific primer 2, 5′-CAG CGA CAC GGC GGG CTC CAT-3′.

Northern blot analysis

Fifteen micrograms of total RNA either from adenoma and the adjacent atrophic gland or from rat adrenal gland were subjected to Northern blot analysis as described previously (19). The membrane containing human RNA was hybridized with full-length hDiminuto cDNA labeled with [α-^{32}P]dCTP (NEN Life Science Products, Boston, MA). The membrane containing rat RNA was hybridized with a 531-bp rat Diminuto (rDiminuto) probe cloned by RT-PCR from rat adrenal total RNA using primers 5′-GAG CTT GAT GAC CTC ACA GTG GGG-3′ (sense) and 5′-GCT GCG CGT GTG GCG GTG GTA GTA-3′ (antisense). The same membranes were rehybridized with rat glyceryldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. The cloning of rat GAPDH was reported previously (19). Analysis of radioactivity was carried out with the BAS 2000 Bioimage analyzing system (Fuji Photo Film Co., Ltd., Tokyo, Japan). hDiminuto and rDiminuto mRNA levels were normalized by GAPDH mRNA levels.

In situ hybridization (ISH)

[^{35}S]UTP-labeled cRNA probe was synthesized by *in vitro* transcription of a pGEM-T Easy plasmid containing the entire coding sequence of hDiminuto cDNA using MAXIscript kit (Ambion, Inc., Austin, TX). The antisense and sense probes used for ISH were transcribed using SP6 and T7 RNA polymerases, respectively, after linearizing the plasmid. Formalin-fixed paraffin-embedded sections were deparaffinized by serial incubations in xylene and graded ethanol. The sections were washed with PBS pretreated with diethylpyrocarbonate (Sigma, St. Louis, MO) and then permeabilized with 0.1% Triton-X in PBS for 10 min at room temperature. After washing with PBS, the sections were postfixed with

4% paraformaldehyde for 5 min. The sections were rinsed in PBS and acetylated with 0.25% acetic anhydride and 0.1 M triethanolamine in saline for 10 min. The sections were washed in graded ethanol. Prehybridization was performed for 3–5 h at 55 C in the prehybridization solution [50% formamide, 10% dextran sulfate, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.1 M Tris-HCl (pH 7.4), 0.4 M NaCl, 5 mM EDTA, 0.1% SDS, 250 μg/ml yeast tRNA, 250 μg/ml salmon sperm DNA, and 100 mM dithiothreitol]. Hybridization was performed in the prehybridization solution containing 1×10^7 cpm/ml ^{35}S-labeled cRNA at 55 C for 16 h. The sections were washed in 50% formamide and 2 × SSC at 55 C and treated with 0.2 μg/ml RNase A (Roche Molecular Biochemicals, Mannheim, Germany) in TNE buffer [0.5 M NaCl, 0.05 M EDTA, and 0.05 M Tris-HCl (pH 7.5)] for 30 min at 37 C. After sequential washing at 20 C with 100 mM dithiothreitol in 2, 1, 0.5, and 0.1 × SSC each for 5 min, the sections were further rinsed twice in 0.1 × SSC and dehydrated with ethanol. The sections were exposed to Kodak x-ray films (Eastman Kodak Co., Rochester, NY) for 7–10 d and dipped into Kodak NTB2 emulsion for 2 wk at 4 C. The slides were developed with Kodak D19, fixed and counterstained with cresyl violet. A serial section was stained with hematoxylin and eosin.

For quantification of grain density for hDiminuto, grains were counted in three fields from the adenoma and adjacent nontumorous gland for each patient. Each field contained similar numbers of cells (~150 cells/field).

In situ apoptosis detection

To detect apoptosis in the adrenal tissues, DNA fragmentation was analyzed by the TUNEL method (20) using an *in situ* apoptosis detection kit from Takara Biomedicals (Tokyo, Japan). Formalin-fixed, paraffin-embedded sections were deparaffinized by serial incubations in xylene and graded ethanol. The sections were washed with water, treated with proteinase K for 15 min, and washed with PBS. After blocking the endogenous peroxidase by 3% H_2O_2 for 5 min, fragmented DNA was labeled with fluorescein isothiocyanate (FITC)-conjugated dUTP in the presence of TdT for 60 min at 37 C. The reaction was terminated by washing with PBS. Anti-FITC-horseradish peroxidase conjugate was used to visualize the incorporated FITC-conjugated dUTP. The sections were incubated with the antibody in the presence of diaminobenzidine and H_2O_2. After washing with water, the sections were counterstained with 3% methyl green. All reagents except H_2O_2, diaminobenzidine, and methyl green were supplied in the kit.

Statistical analysis

Statistical analysis was carried out using one-way ANOVA, followed by Fisher's protected least significant difference analysis.

Results

After subtractive hybridization, several cDNAs, selectively up-regulated in the adenoma, were identified by the screening with Southern blotting. Cloning of the cDNAs and subsequent sequence analysis revealed that most of the clones were independent, suggesting the efficacy of subtractive hybridization. A BLAST (http://www.blast.genome.ad.jp) search revealed that the nucleotide sequence of one clone of 555 bp was completely identical to the region from 3633–4187 bp of KIAA0018 cDNA (GenBank accession no. D13643), which is the human homolog of the Diminuto/Dwarf1 gene described in plants and *Caenorhabditis elegans*. This sequence also matches exactly the region from 3694–4248 bp of seladin-1 cDNA (GenBank accession no. AF261758). In both cases the isolated sequence corresponds to the 3′-most end of the cDNAs. The flanking *Rsa*I sites at both ends reside in the adaptor oligonucleotides used for cDNA synthesis.

KIAA0018 cDNA was isolated from human immature myeloid cell line KG-1 (21). The existing cDNA sequence in GenBank database does not contain information about the 5′-most end of the cDNA or translation start site. Seladin-1 was isolated from human brain, and the cDNA sequence is 61 bp longer than KIAA0018, showing the complete coding sequence. We checked whether the cDNA sequence is identical and whether some other isoforms of the molecule exist in human adrenocortical cells. Based on the existing sequences, we cloned the cDNA from normal human adrenal gland by RT-PCR and 5′-RACE. Our findings revealed that the cDNA sequence is completely identical to the seladin-1 sequence, and there is no other isoform. The cDNA codes for a protein of 516 amino acids.

Next, we looked for the chromosomal localization of this gene. BLAST search identified the whole sequence in *Homo sapiens* clone RP11−12 C17 (accession no. AC009946, version AC009946.2), which contains continuous 169,072-bp sequence present in chromosome 1. The genomic sequence of hDiminuto lies in this clone in reverse orientation spanning 37,487 bp. The gene is encoded by nine exons. All of the exon-intron junctions follow the GT/AG rule. The coding sequence starts from base 100 of exon 1 and ends at base 151 of exon 9. Exon 9 contains the entire 3′-untranslated region, including the polyadenylation signal. The genomic organization of hDiminuto is shown in Fig. 1. A search for the cytogenetic map in NCIB (National Center for Biotechnology Information, National Library of Medicine and National Institute of Health) revealed that the gene is localized to chromosome 1p32.

In the screening procedure hDiminuto showed a markedly high difference in the expression level between the adenoma and the adjacent atrophic tissue. We confirmed the authenticity of the preferential expression of hDiminuto in the adenoma by Northern blot analysis. As shown in Fig. 2A, hDiminuto mRNA was detected as a single band of 4.2 kb, which corresponds to the size expected from the cDNA sequence. In 14 patients studied, hDiminuto mRNA was abundantly expressed in the adenomas, whereas its expression was either low or undetectable in the adjacent atrophic nontumorous glands. In contrast, the expression of the housekeeping gene GAPDH was similar in these two tissues.

We strengthened our observation of Northern blot analysis further by ISH on formalin-fixed paraffin-embedded

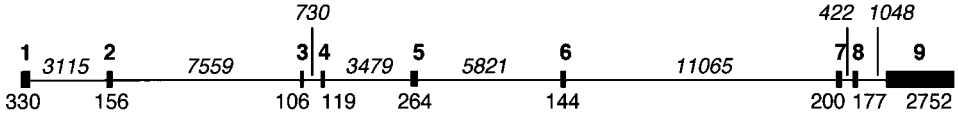

FIG. 1. Genomic organization of hDiminuto gene. *Black boxes* and *horizontal lines* represent the exons and introns, respectively. The numbers in *bold* represents exon number. The numbers in *italics* denote the sizes of the introns in base pairs. Other numbers denote the sizes of the exons in base pairs.

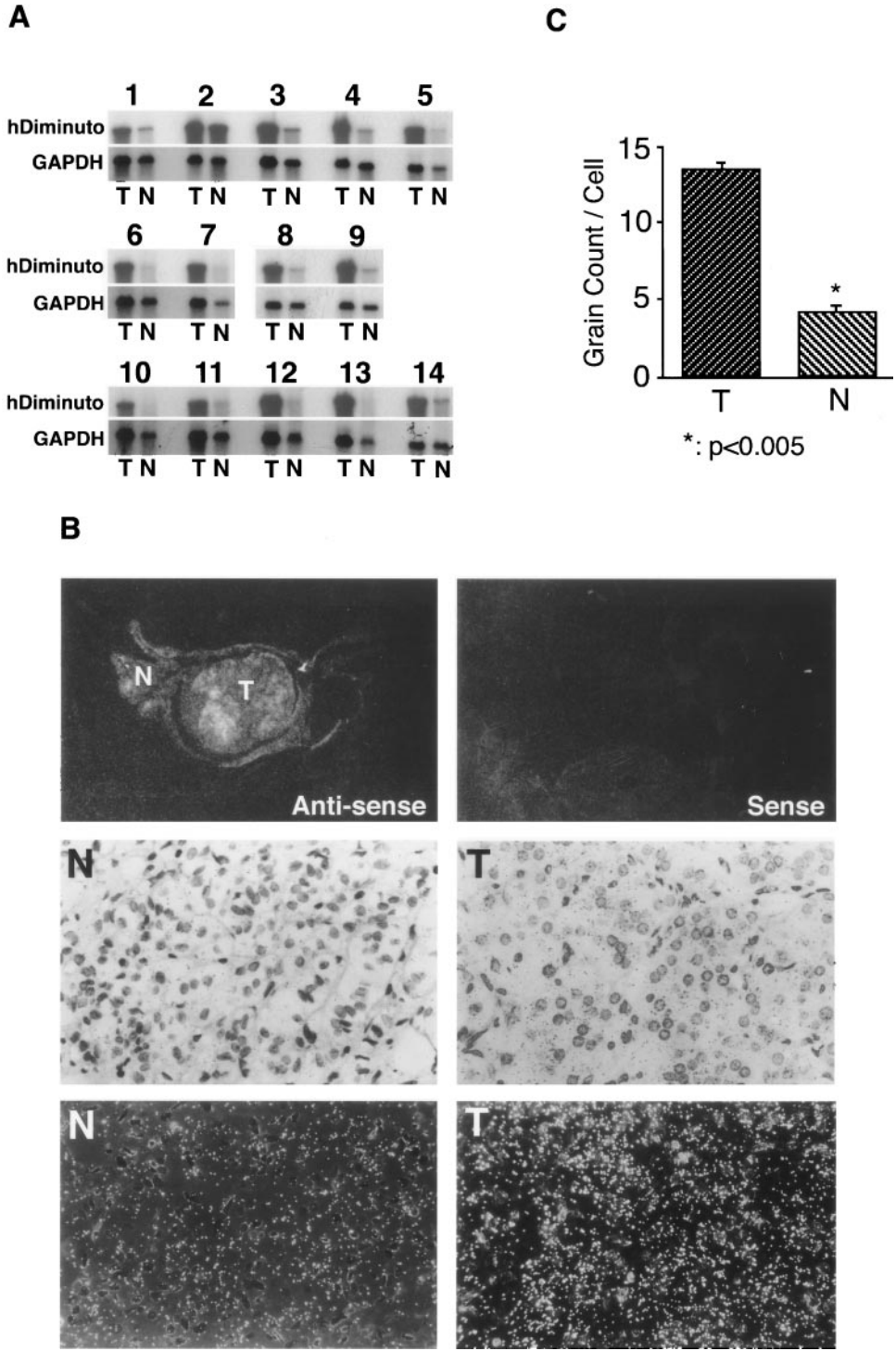

FIG. 2. hDiminuto mRNA is expressed more in the adenoma (T) than in the adjacent nontumorous adrenal gland (N). A, Expression of hDiminuto and GAPDH mRNAs in the adenoma and adjacent nontumorous gland was determined by Northern blot analysis as described in *Subjects and Methods*. The numbers denote the patient numbers. B, Formalin-fixed paraffin-embedded sections containing both the adenoma and the adjacent adrenal gland were hybridized with antisense (*upper left panel*) and sense (*upper right panel*) hDiminuto cRNA probes. The *middle panel* shows brightfield and the *lower panel* shows darkfield microscopic views of the section hybridized with antisense probe at ×500 magnification. C, The hDiminuto grain number in adenoma *vs.* adjacent nontumorous gland was counted. Data represent the mean ± SD of grain counts from three fields containing similar numbers of cells (~150 cells/field). *, $P < 0.005$.

sections. The *upper left panel* in Fig. 2B shows a macroscopic view of a section containing both the adenoma and the adjacent atrophic nontumorous gland. Note that no expression could be detected when hybridization was carried out with the sense probe (*upper right panel*). Low level, uniform expression of hDiminuto mRNA could be detected in the adjacent nontumorous gland with the antisense probe. In the adenoma, hDiminuto mRNA was abundantly expressed in the tumor cells. *Middle* and *lower panels* show bright- and darkfield microscopic views of the same section, respectively. Darkfield views clearly demonstrated abundant expression of the mRNA in the adenoma compared with the adjacent nontumorous gland. Quantification of the expression level by counting the grains revealed that the grain count per cell was about 3.5-fold higher in the adenoma than in the adjacent gland (Fig. 2C).

As the adjacent atrophic gland may not represent the normal architecture, we studied the expression pattern of hDiminuto mRNA in normal human adrenal gland by ISH. The adrenal glands were obtained from patients operated for nonadrenal pathologies, such as paraganglioma, neurinoma of the retroperitoneal space, and adrenal cyst. The *left panel* in Fig. 3 depicts a section of the normal human adrenal cortex stained with hematoxylin/eosin showing all of the layers. The *right panel* depicts a serial section hybridized with the hDiminuto cRNA probe. hDiminuto mRNA expression could be detected in all layers of adrenal cortex. Abundant expression was detected in zona fasciculata.

The recent finding that the product of hDiminuto serves as a substrate for the apoptosis-related endoproteolytic cleavage enzyme, caspase, and protects neuronal cell death from oxidative stress (17) prompted us to examine whether hDiminuto expression could be correlated with the degree of apoptosis. TUNEL staining of a serial section from the normal adrenal gland shown in Fig. 3 revealed that little, if any, apoptotic signals was detected (Fig. 4A). In contrast, many TUNEL-positive cells were observed in the atrophic adrenal gland adjacent to the adenoma (Fig. 4B). Of particular interest was the fact that almost no apoptotic signal was detected in the adenoma (Fig. 4C), where hDiminuto expression was much higher than in the adjacent atrophic gland (Figs. 2 and 3).

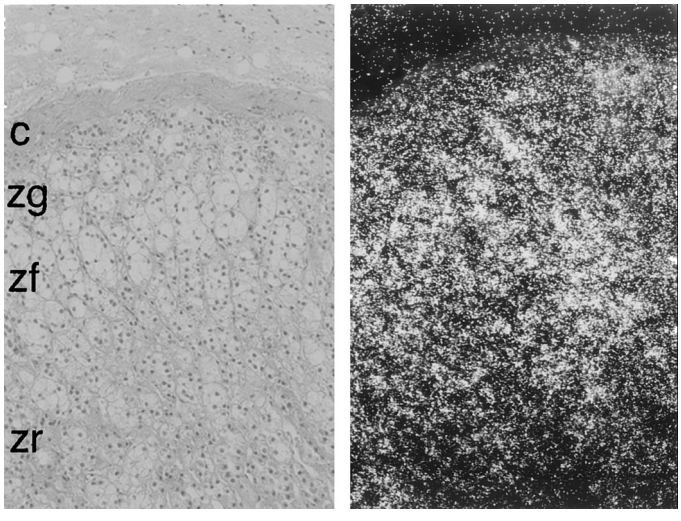

FIG. 3. Expression of hDiminuto mRNA in normal human adrenal gland. A formalin-fixed paraffin-embedded section was hybridized with antisense hDiminuto cRNA probe. The *right panel* shows a darkfield microscopic view at ×200 magnification. The *left panel* shows a serial section stained with hematoxylin/eosin. c, Capsule; zg, zona glomerulosa; zf, zona fasciculata; zr, zona reticularis.

Abundant expression of hDiminuto in zona fasciculata in the normal adrenal cortex suggested its physiological role in cortisol biosynthesis and its possible regulation by ACTH/cAMP. We, therefore, checked whether the expression of hDiminuto mRNA could be modulated by activating the cAMP signaling pathway. We treated H295R human adrenocortical carcinoma cells with the adenylate cyclase activator, forskolin (10^{-5} mol/liter), for up to 48 h. As shown in Fig. 5A, hDiminuto mRNA had high basal expression in H295R cells. The expression level started increasing at 12 h after treatment with forskolin, and at 48 h it showed more than 2-fold induction. We also checked the dose-dependent induction of hDiminuto mRNA by forskolin. As shown in Fig. 5B, treatment with 10^{-11}–10^{-7}mol/liter forskolin for 24 h did not induce the mRNA. However, treatment with 10^{-5} mol/liter forskolin for 24 h resulted in a 2-fold induction.

As we employed a carcinoma cell line for the *in vitro* experiment, we performed an *in vivo* experiment to study the physiological regulation in rats. Male Wistar rats were treated with dexamethasone to suppress endogenous ACTH secretion. Then the rats were treated with ACTH. As shown in Fig. 6, abundant expression of rDiminuto mRNA was detected in the control group. Treatment with dexamethasone for 3 d resulted in a 4-fold reduction in the expression level. Subsequent treatment with ACTH resulted in an increase in the expression level of rDiminuto at 12 h. At 48 h the expression level was similar to that in the control group, indicating that ACTH is required for the normal level of

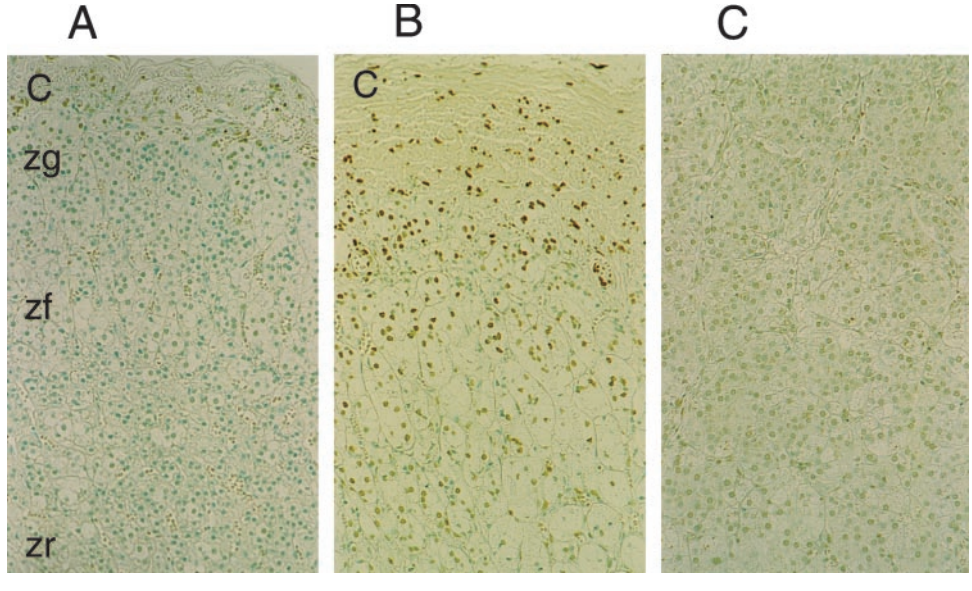

FIG. 4. Diminished expression of hDiminuto is associated in an increase in the number of TUNEL-positive cells in the adjacent nontumorous gland. TUNEL staining of sections from normal adrenal (A), atrophic nontumorous gland adjacent to the adenoma (B), and adenoma (C) is shown. Note that almost no TUNEL-positive cells were observed in normal adreanl or adenoma, where the expression of hDiminuto was abundant (see Figs. 2 and 3). In contrast, numerous TUNEL-positive cells were observed in the adjacent nontumorous gland (B), where hDiminuto expression was diminished. c, Capsule; zg, zona glomerulosa; zf, zona fasciculata; zr, zona reticularis.

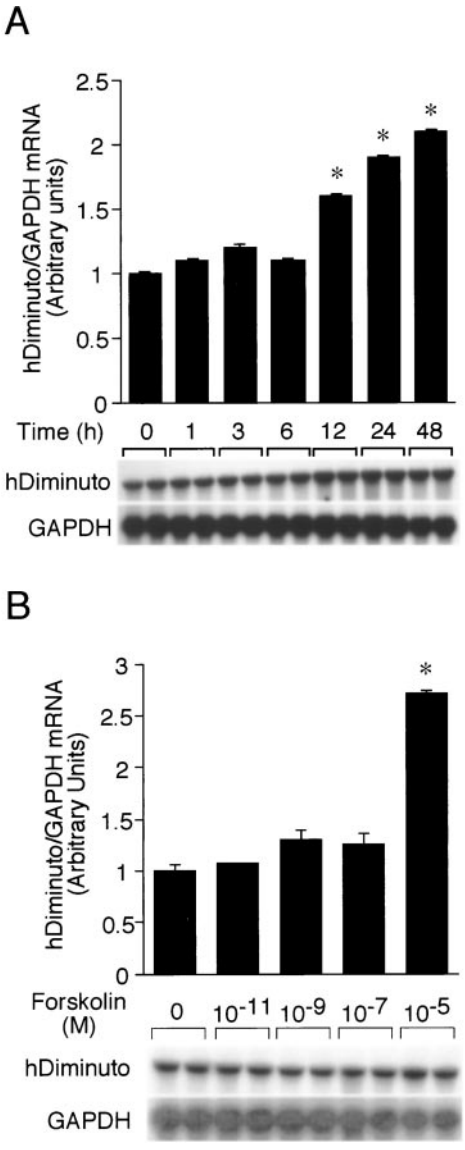

FIG. 5. Forskolin up-regulates hDiminuto mRNA expression. A, H295R cells were treated with 10 μmol/liter forskolin for the indicated time periods. Expression of hDiminuto and GAPDH mRNAs in these cells was determined by Northern blot analysis as described in *Subjects and Methods*. *, Significant differences from 0 h ($P < 0.01$). B, H295R cells were treated with indicated concentrations of forskolin for 24 h. Expression of hDiminuto and GAPDH mRNAs in these cells was determined by Northern blot analysis as described in *Subjects and Methods*. *, Significant difference from 0 M forskolin ($P < 0.01$). The experiments were carried out twice. The data represent the mean ± SD.

expression. ACTH treatment did not induce mRNA expression further.

Discussion

In this report we demonstrate for the first time that hDiminuto mRNA is overexpressed in benign adrenocortical adenomas, and the expression is suppressed in the adjacent nontumorous adrenal gland. These contrasting expression patterns of hDiminuto in these tissues are similar to that of ACTH receptor, as we reported previously

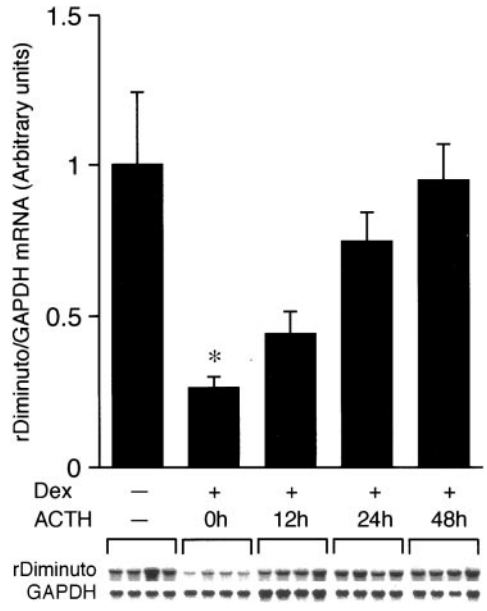

FIG. 6. ACTH positively regulates rDiminuto mRNA expression in rats. Male Wistar rats were treated either with dexamethasone alone or subsequently with ACTH as described in *Subjects and Methods*. Expression of rDiminuto and GAPDH mRNAs in the adrenal glands was determined by Northern blot analysis. *, Significant difference *vs.* control and ACTH-treated groups ($P < 0.01$).

(3). Thus, one possible explanation for the overexpression of hDiminuto in the adenoma is the abundant expression of ACTHR. This possibility was tested by *in vitro* and *in vivo* experiments to check whether forskolin or ACTH regulates hDiminuto expression. Indeed, hDiminuto mRNA expression was demonstrated to be positively regulated by ACTH/cAMP, suggesting its physiological role in the function of adrenocortical cells.

There are two physiological processes that are augmented in adrenocortical adenomas, cell growth and steroid synthesis. It is possible that hDiminuto is important for both of these functions. We observed that the expression level was down-regulated in the adjacent nontumorous gland in patients with CS and also in dexamethasone-treated rats. In both cases the adrenal glands were atrophied, suggesting that hDiminuto might be involved in the growth of adrenocortical cells. Analysis of the amino acid sequence of hDiminuto has revealed that it contains a domain characteristic of oxidoreductases that harbors a consensus sequence for noncovalent flavin adenine dinucleotide (FAD) binding (22, 23). FAD-dependent enzymes catalyze reduction-oxidation reactions in which the FAD moiety accepts one or two electrons from a reduced substrate and donates one or two electrons to the oxidized substrate of an electron acceptor (24). They are integral parts of the intracellular metabolic pathways including steroidogenesis and detoxification of endogenous compounds and xenobiotics (24). During the process of steroidogenesis, lipid peroxides and other free oxygen radicals are generated (25). As an oxidoreductase, hDiminuto might be required to detoxify these molecules to protect the cells. In both rat and human adrenal glands as well as in H295R cells, the basal expression level of the mRNA was quite high.

Among the human tissues studied, the adrenal gland showed the maximum level of expression (17). It has been reported that even in the absence of active steroid synthesis there is significant electron leakage from the steroid synthetic enzyme system leading to the production of reactive oxygen radicals (26). High basal hDiminuto expression thus might be necessary to protect the cells from oxidative damage. Another function of hDiminuto could be the detoxification of toxic by-products of steroid hormone synthesis. Mouse vas deferens protein, an aldose reductase-like protein, neutralizes isocaproaldehyde, a toxic product of side-chain cleavage of cholesterol (27). Inhibition of mouse vas deferens protein results in increased cell death upon treatment with forskolin as a result of isocaproaldehyde accumulation. hDiminuto might also be involved in a similar function. These hypotheses are substantiated by the fact that hDiminuto is overexpressed in adrenocortical adenomas actively synthesizing steroid. hDiminuto (seladin-1) confers resistance to Alzheimer's disease-associated neurodegeneration and oxidative stress (17). It protects the neurons against amyloid-β peptide or hydrogen peroxide-induced toxicity and thus acts as an antiapoptotic factor. In plants Diminuto is necessary for cell growth (16). The present study demonstrated that the level of hDiminuto expression was inversely correlated with the number of TUNEL-positive cells *i.e.* its diminished expression is associated with abundant apoptotic cells. These findings indicate that hDiminuto has an important role in cell survival and sustenance.

Another possible function of hDiminuto might be its direct involvement in steroid synthesis. The subcellular localization of hDiminuto (seladin-1) has been shown to be the endoplasmic reticulum (17). Interestingly, Diminuto is located in the endoplasmic reticulum in plants also (15). In the endoplasmic reticulum of adrenocortical cells, three major enzymes, 3β-hydroxysteroid dehydrogenase, 17α-hydoxylase, and 21-hydroxylase, catalyze cortisol biosynthesis (28). Both 17α- and 21-hydroxylases require NADPH cytochrome P450 reductase and cytochrome b5 for the purpose of electron transfer and subsequent substrate oxidation (28). Cytochrome b5 greatly facilitates the 17,20-lyase activity of 17α-hydroxylase, which leads to the production of androgens (29). Overexpression of cytochrome b5 has been demonstrated in androgen-producing adrenocortical adenomas (30). In a similar fashion, overexpression of hDiminuto in cortisol-producing adenomas indicates that it might be involved in cortisol biosynthesis. Our ISH findings revealed abundant expression of hDiminuto mRNA in zona fasciculata, the principal site of cortisol biosynthesis. As a putative FAD- and NADP-binding oxidoreductase, hDiminuto might actively take part in this synthetic pathway.

These speculations about the function of hDiminuto correlate with the fact that its expression is increased by ACTH. Simultaneous augmentation of steroidogenic enzymes and hDiminuto by ACTH might facilitate steroidogenesis without compromising cell viability. Studies are in progress to substantiate these speculations and identify the function of hDiminuto in normal human adrenal gland.

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