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PRINCIPAL INVESTIGATOR: Jer-Tsong Hsieh, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Southwestern
Medical Center at Dallas
Dallas, TX 75390-9105

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Jer-Tsong Hsieh, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas Southwestern
Medical Center at Dallas
Dallas, Texas 75390-9105

E-Mail: jhsieh@mednet.swmed.edu

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It is believed that androgen independent (AI) prostate cancer (PCa) cells behave like the basal cells in prostate gland with malignant phenotype. Basal cells represent a stem cell population of prostate. Therefore, we believe that a genetic imbalance for controlling growth and/or differentiation in basal cell will result in the onset of AIPCa. Our previous studies discover a novel protein-DOC-2 that are associated with normal basal cell of prostate and are often absent in prostate cancer. Most importantly, we identified a novel RAS-GTPase activating protein (i.e., DIP1/2) interacting with DOC-2 protein. In this project, we have studied the regulation of DIP1/2 in prostate cancer cells and signal transduction of DOC-2/DIP1/2 complex during cell differentiation of prostatic epithelium.

In summary, we demonstrated that epigenetic control such as DNA methylation and histone acetylation plays an important role in modulating DIP1/2 gene expression in AIPCa. The re-expression of DIP1/2 was able to suppress the growth of PCa cells. Using a three-dimension *in vitro* tissue culture system, the elevated DIP1/2 protein and several other genes (such as P-cadherin, prostate-derived factor) correlated with the cell polarization of basal epithelium. Furthermore, DIP1/2-expressing cells can form glandular tissue *in vivo*. Thus, DIP1/2 is a critical factor involved in cell differentiation of prostatic epithelium.

14. SUBJECT TERMS

Stem cell, androgen-independent growth, tumor suppressor, adaptor protein, cell adhesion molecule, prostate cancer

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INTRODUCTION

Prostate gland is an androgen-dependent organ, particularly, prostatic epithelia. Morphologically, prostatic epithelia can be divided into luminal epithelia (androgen-dependent differentiated cell) and basal epithelium (androgen-independent [AI] poorly differentiated cell). Although the cell lineage of prostatic epithelia is not well characterized, it is postulated that basal cell, a progenitor for luminal epithelial cells (1-4), are considered as the stem cell because it is responsible for maintaining the homeostasis of normal prostate. In contrast, AI prostate cancer (PCa), a life threatening disease resulting in the major mortality rate of this disease, exhibits several unique proteins as seen in basal cells (5-6), suggesting that AI PCa possesses stem cell characteristics plus malignant phenotype. Such malignant phenotype may be due to the loss of homeostatic control in these cancer cells.

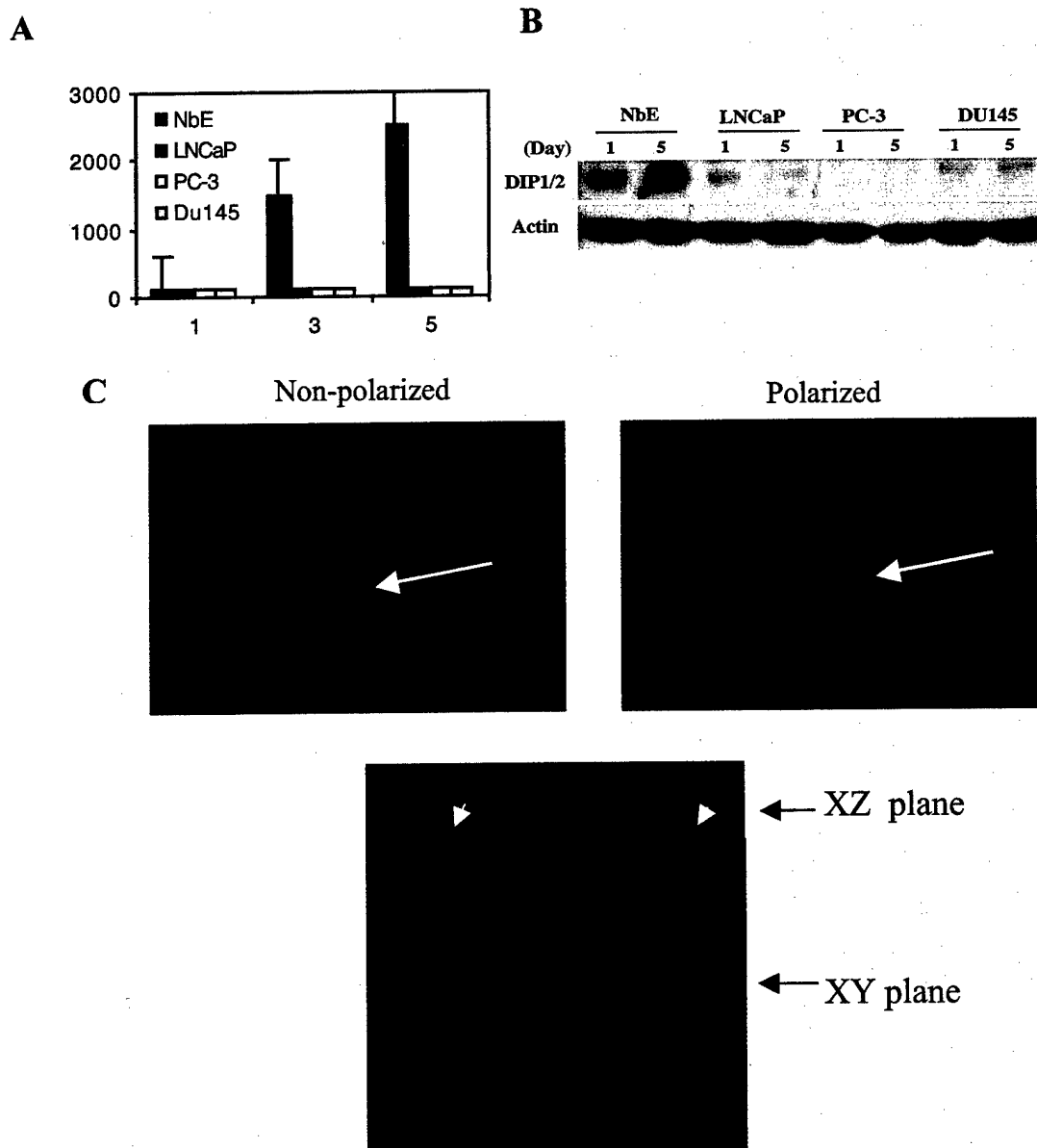
By delineating the biology and function of these basal cells, we could unveil the defect of AI PCa, which could help to formulate new strategy of therapy. To achieve this goal, we obtained normal basal prostatic epithelia from normal prostate and characterized their differentiation potential from *in vitro* and *in vivo*. Using differential display RT-PCR and cDNA microarray, we also discovered novel proteins that may be involved in the cell differentiation of basal cells. The outcome of this project has provided new information for understanding cell differentiation of prostatic epithelium based on molecular biologic analyses. This knowledge should not only provide new diagnostic marker(s) for AIPCa also help to invent new therapeutic regimes for this disease.

BODY

Task 1. To isolate different population of prostatic epithelia based on these three markers and characterize the morphologic changes associated with these cells from *in vitro*

In order to characterize the differentiation potential of basal cell isolated from normal prostate, we employed a three-dimension culture system. A defining characteristic of polarized epithelium, a hallmark of a differentiated epithelium, is their ability to seal paracellular space and maintain the distinct adjacent tissue compartments. Tight junction complex is a continuous circumferential intercellular contact at the apical pole of lateral cell membrane (7, 8). In contrast, cancer cells often lose their polarized phenotype as well as tissue architecture as a result of dedifferentiation. We found that normal epithelial cell such as NbE could form tight junction when it grew in a Transwell® measured by an increased Transepithelium Resistance (TER) (Fig. 1A). Both Actin and ZO-1 staining showed a defined pattern in polarized pattern in polarized NbE compared to non-polarized NbE using fluorescence microscopy. However, several PCa cell lines failed to form any tight junction (Fig. 1A). As shown in Fig. 1B, an elevated DIP1/2 level was consistent with the formation of tight junction in NbE cell. Also, the ZO-1 protein (Fig. 1C), a key component in tight junction, exhibited a distinct network structure in polarized NbE cells but not in polarized NbE cells. Indeed, ZO-1 was localized in the tight junction of polarized NbE cells using confocal microscope. This data indicate that NbE can undergo differentiation *in vitro* and DIP1/2 may be involved in this process.

Fig. 1 Increased levels of DIP1/2 during cell polarization of prostatic epithelia. Each cell line (3×10^5 cells/well) was plated in a Transwell® (12-mm). At the indicated time, TER was determined (A) and cell lysate was subjected to western blot analysis (B). The immunostaining of ZO-1 protein in non-polarized and polarized NbE cells (C).



As we noticed that the elevated DIP1/2 levels were detected in cell polarization and decreased DIP1/2 were detected in PCa cells, we decided to examine the possible regulatory mechanism in DIP1/2 gene. Since epigenetic control such as DNA hypermethylation and/or histone deacetylation can lead to transcription repression that is often associated with a number of tumor suppressor gene promoters, including Rb, p15 and p16 (9, 10). Noticeably, the 5'-upstream sequence of hDIP1/2 is also very GC-rich and many CpG islands were identified. We decided to determine whether the hypomethylation agent, 5-azacytidine (5-Aza), has any effect on the regulation of hDIP1/2 gene. Also, we examined the effect of histone deacetylase (HDAC)

inhibitor such as Trichostatin A (TSA) on the expression of hDIP1/2 gene. Either 5-Aza or TSA alone can induce endogenous hDIP1/2 mRNA expression in both LNCaP and PC-3 cells but not in HPV-PZ-7 (see Appendix 1). Furthermore, single or combination treatment of both drugs exhibited an additive effect on the hDIP1/2 gene promoter activities of hDIP1/2 gene in both LNCaP and PC-3 cells (Fig. 5 and Table 2 of Appendix 1). Using both ChIP assay and bisulfite DNA sequencing (Figs. 4 and 6 of Appendix 1), we were able to correlate the status of acetyl-histone and DNA methylation with hDIP1/2 promoter region activity in PCa cell lines. These data indicate that both DNA hypermethylation and histone deacetylation are critical to modulate the expression of DIP1/2 gene.

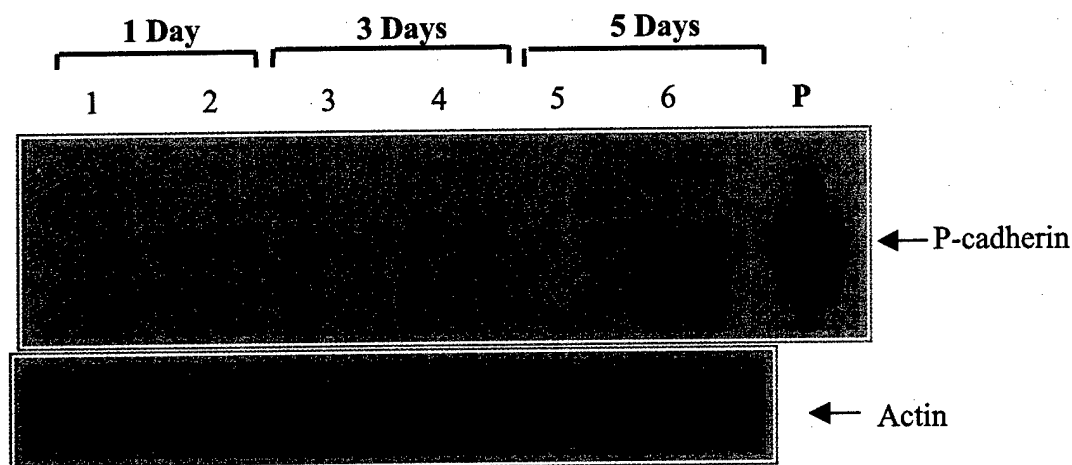
In addition, we searched other genes involved in cell polarization process using 22K cDNA array. As shown in Table 1, we have found 14 potential genes upregulated in polarized cell from two rounds of screening. To validate the data generated from microarray, we performed real time RT-PCR for each gene and we only confirmed the increased cadherin 3 (i.e., P-cadherin) in polarized cell. As shown in Fig. 2, the steady-state levels of P-cadherin were elevated in polarized cells in a time-dependent manner. P-cadherin is known to be a basal cell specific marker in prostate and the absent expression of P-cadherin is often detected in PCa (11). Therefore, based on our results, we believe that P-cadherin may play a homeostatic role in controlling cell differentiation.

Table1 Summary of up-regulated genes in polarized cells obtained from microarray screening

Gene ID	Gene description	Fold of induction*	
		Experiment 1	Experiment 2
NM001793	Cadherin 3	3.96	2.07
NM012142	Cyclin D-type binding protein 1	5.69	2.51
NM001967	Eukaryotic translation initiation factor 4A	2.43	2.07
NM002026	Fibronectin	3.02	4.75
AK0555564	FL22182 fis	6.35	3.48
NM002165	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	2.07	2.51
NM000228	Laminin, γ 2 (LAMC2) transcript variant	5.01	2.95
NM002391	Laminin, β 3 (LAMB3)	2.78	2.09
NM013597	Midkine (neurite growth-promoting factor 2)	3.88	2.27
NM004864	Neuregulin1	2.16	2.00
NM030666	Prostate differentiation factor	3.99	3.00
NM004753	Serine (or cysteine) proteinase inhibitor CladB	2.84	3.97
NM003380	Short-chain dehydrogenase/ reductase 1	5.92	2.48
NM003380	Vimentin	2.22	2.06

*Fold of induction is calculated using GeneSpring software.

Fig. 2 Determination of the steady-state levels of P-cadherin protein in prostatic epithelium during cell polarization. Each cell line (3×10^5 cells/well) was plated in a Transwell® (12-mm). At the indicated time, cells were harvested and cell lysate was subjected to western blot analysis probed with P-cadherin antibody. 1, 3, 4 : PC3 cells; 2, 4, 6: NbE cells; P: positive control for P-cadherin.



Task 2. To determine the stem cell potential of basal cell population from *in vivo*.

It is known that the role of stroma is crucial for the morphogenesis of glandular epithelium and even cancer progression (13-15). In order to study any molecule event involved in prostatic epithelial differentiation, the contribution of stromal must be considered. Although, our *in vitro* data indicated that the potential role of DIP1/2 in cell polarization using an *in vitro* system, these data have to be validated using an *in vivo* model. We decided to employ the subrenal capsule grafting model (13, 14) that has been successfully used in studying the effect of epithelial and stromal interaction on glandular formation as well as cancer progression.

In order to make chimeric organ, inductive fibroblast- urogenital sinus mesenchyme (UGM) was prepared from E16 embryo by isolating urogenital sinus (UGS) and removing urogenital sinus epithelia from UGS. After collagenase digestion, the UGM became single cell suspension, and then mixed with epithelia in the presence collagen for implantation. As shown in Fig. 3A, NbE cells were mixed with UGM cells and a distinct glandular formation was detected 6 weeks after implantation. This model provides a unique system to implant *in vitro* genetically manipulated cell and observe the *in vivo* morphologic change. To examine whether DIP1/2 expression may impact on the glandular formation of prostate, we generated stable transfectant of PC-3 expressing DIP1/2 vector. By implanting these cells into subrenal capsule, we were able to observe the formation of glandular tissue after 3 months (Fig. 3B). In order to delineate the potential marker(s) associated with cell differentiation of PC-3 *in vitro*, we carefully dissect tissue from chimeric organ using microdissection and performed microarray screening. We analyzed these tissues for its gene expression profile (Table 2), we only found only 10 genes differentially expressed in chimeric organ from two round of screening. Currently, we are validating these genes.

Fig. 3 The impact of DIP1/2 protein on the ductal formation of prostatic epithelia using renal capsule grafting model. A, NbE (25,000 cells) plus UGM cells (100,000) mixed in collagen gel and implanted under subrenal capsule for 6 weeks. B, DIP1/2-PC-3 (20,000 cells) plus UGM cells (100, 000) mixed in collagen gel and implanted under subrenal capsule for 3 months. K, kidney; L, lumen; LE, luminal epithelia; BE, basal epithelia, S, stroma.

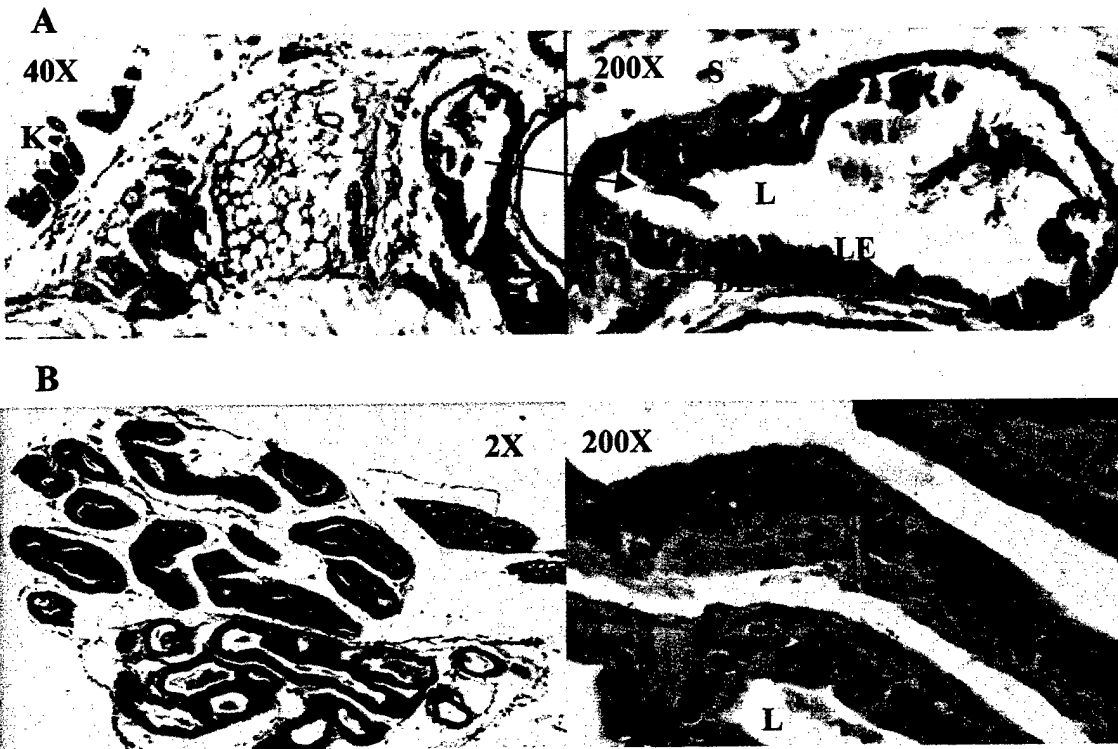


Table 2 Summary of differential gene expression in chimeric organ screened by microarray

Gene ID	Gene description	Fold of induction*	
		Experiment 1	Experiment 2
NM005213	Cystatin A (Stefin A)	10.59	2.38
NM000772	CytochromeP450, family 2 subfamily C, polypeptide 18	4.73	3.63
AK054598	FLJ30036fis	4.28	4.23
NM000223	Keratin 12	2.46	2.37
NM005557	Keratin 16	3.71	4.06
BC009699	Metallothionine 1E	2.03	2.06
NM005607	PTK2 protein tyrosine kinase	2.17	4.88
NM002964	S100 Calcium binding protein A8 (Calgraulin A)	6.03	3.71
NM005416	Small proline-rich protein 3	2.24	3.29
S73288	Small proline-rich protein SPRK	5.31	5.69

*Fold of induction is calculated using GeneSpring software.

KEY RESEARCH ACCOMPLISHMENT

- Isolate three different subpopulation of prostatic epithelium from degenerated prostate based on C-CAM1 expression and characterize each clone of epithelia using several different makers.

- Determine the differentiation potential (i.e., cell polarization) of these cells using a three-dimension cell culture system.
- Identify other molecules differentially expressing in polarized cell and non-polarized cell population.
- Characterize the homeostatic role of DOC-2 protein in the growth of prostatic epithelia.
- Identify the functional role of DIP1/2 protein in the growth/differentiation of PCa.
- Clone human DIP1/2 gene and demonstrate the epigenetic control of this gene in normal prostatic epithelia and PCa cells.
- Establish a three-dimension cell culture system for studying cell differentiation of normal prostatic epithelium.
- Screen potential markers associated with the process of cell polarization of prostatic epithelia using cDNA microarray.
- Demonstrate the role of DIP1/2 protein in glandular formation of prostate using renal capsule grafting technique.

REPORTABLE OUTCOMES

FULL-LENGTH PAPER

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4. Chen, H., Toyooka, S., Gazdar, A.F., and Hsieh, J.T. (2003) Epigenetic regulation of a novel tumor suppressor gene (hDAB2IP) in prostate cancer cell lines. *J. Biol. Chem.*, 278, 3121-3130.
5. Zhou, J., Scholes, J., and Hsieh, J.T. (2003) Characterization of a novel negative regulator (DOC-2/DAB2) of c-Src in normal prostatic epithelium and cancer. *J. Biol. Chem.*, 278, 6936-6941.

5. Zhou, J., Scholes, J., and Hsieh, J.T. (2003) Characterization of a novel negative regulator (DOC-2/DAB2) of c-Src in normal prostatic epithelium and cancer. *J. Biol. Chem.*, 278, 6936-6941.

REVIEW PAPER

1. Zhou, J., Scholes, J., and Hsieh, J.T. (2001) Signal transduction targets in androgen independent prostate cancer. *Cancer and Metastasis Review*, 20: 351-362.

CONCLUSIONS

We hypothesize that the basal cells of the prostate gland, considered as a stem cell population, are responsible for maintaining homeostasis of the normal prostate. We have isolated basal cells from normal prostate and demonstrated that these cells were able to form tight junction of polarized cell *in vitro*. These cells should represent the progenitor for glandular epithelia. Using this *in vitro* model, we were able to unveil the molecular marker(s) associated with polarized cells and further study the possible functional role of these marker(s).

To understand the cell differentiation of prostatic epithelia, we found that DIP1/2 was associated with polarized epithelium. DIP1/2 is new member of RAS-GTPase activating protein family that is known to modulate extracellular signal-elicited RAS pathways. We found that DIP1/2 was elevated in polarized cell and the regulation of this gene was due to epigenetic control such as DNA methylation and histone acetylation. On the other hand, increase DIP1/2 expression in PC-3 cells can restore the differentiation potential of this cancer cell line. Thus, DIP1/2 and its interactive protein DOC-2 (Appendix 2 and 3) is a homeostatic protein complex controlling cell differentiation of prostatic epithelia.

In addition, using microarray screening, we obtained several candidate genes with upregulation from polarized cells. Among them, we were able to confirm the elevation of P-cadherin in polarized cell in a time-dependent manner. Since P-cadherin is a basal cell specific cadherin and its expression is often undetectable in PCa, our data provide further evidence that basal cell is the progenitor for luminal epithelium. Thus, further investigation of these markers will certainly help us to map the pathway of prostatic epithelial differentiation and provide new knowledge for the defects in AIPCa cells.

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Epigenetic Regulation of a Novel Tumor Suppressor Gene (*hDAB2IP*) in Prostate Cancer Cell Lines*[§]

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Hong Chen[‡], Shinichi Toyooka[§], Adi F. Gazdar^{§1}, and Jer-Tsong Hsieh^{‡1}

From the Departments of [‡]Urology and [§]Pathology and the [§]Hamon Center for Therapeutic Oncology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9110

***hDAB2IP* (human *DAB2* (also known as *DOC-2*) interactive protein) is a novel GTPase-activating protein for modulating the Ras-mediated signal pathway. We demonstrate that the down-regulation of *hDAB2IP* mRNA in prostate cancer (PCa) cells is regulated by transcriptional levels. Analysis of the *hDAB2IP* promoter revealed that it is a typical TATA-less promoter containing many GC-rich sequences. In this study, we delineated the potential impact of the epigenetic control of the *hDAB2IP* promoter on its gene regulation in PCa. Acetylhistone H3 was associated with the *hDAB2IP* promoter, and CpG islands remained almost unmethylated in normal prostatic epithelia, but not in PCa cell lines. Our data further indicated that trichostatin A (histone deacetylase inhibitor) and 5'-aza-2'-deoxycytidine (DNA hypomethylation agent) acted cooperatively in modulating *hDAB2IP* gene expression in PCa, whereas histone acetylation played a more significant role in this event. Moreover, a core promoter sequence from the *hDAB2IP* gene responsible for these treatments was identified. We therefore conclude that epigenetic regulation plays a potential role in regulating *hDAB2IP* expression in PCa and that these results also provide a new therapeutic strategy for PCa patients.**

hDAB2IP (human *DAB2* interactive protein) is a novel member of the Ras GTPase-activating family (1, 2). Our recent data indicate that it interacts directly with *DAB2* (*Disabled-2*; also known as *DOC-2* for differentially expressed in ovarian carcinoma-2) (2), which appears to be a tumor suppressor in cancer types (3–7). Both *DAB2IP* and *DOC-2*/*DAB2* form a unique protein complex with negative regulatory activity that modulates the Ras-mediated signal pathway (2). In the prostate gland, this complex is detected in the basal cell population (2, 6) and may orchestrate the differentiation and proliferation potential of these cells during gland development. In contrast, loss of expression of *DOC-2*/*DAB2* and *hDAB2IP* proteins is often detected in metastatic pros-

tate cancer (PCa)¹ cell lines, and increased expression of these proteins can suppress the growth of PCa (2, 6).

We have demonstrated that normal prostatic epithelial cells have elevated *hDAB2IP* mRNA levels compared with PCa cells, which correlate with increased *hDAB2IP* promoter activity (1). These data indicate that transcriptional regulation of *hDAB2IP* is responsible for the down-regulation of *hDAB2IP* expression in PCa cells. However, little is known about the underlying mechanisms for the regulation of *hDAB2IP* gene expression in prostatic epithelial cells.

One of the hallmarks of the regulation of gene transcription is local chromatin decondensation mediated by histone acetylation, which leads to a reduced association between chromosomal DNA and histones and subsequently increases the accession of high molecular mass protein complexes of the transcription machinery. Conversely, histone deacetylation can repress transcription by increasing histone-DNA interaction (8, 9). Additionally, we have found that the *hDAB2IP* promoter does not have a typical TATA box, but contains many GC-rich sequences (1, 2). DNA hypermethylation, particularly in the GC-rich promoter region, results in transcription repression that is often associated with a number of tumor suppressor gene promoters, including *Rb*, *p15*, and *p16* (10, 11). In this study, we delineated the roles of histone acetylation and DNA methylation in the regulation of the *hDAB2IP* gene in normal prostatic epithelia and PCa cells. The data presented in this work provide strong evidence for underlying mechanisms of the down-regulation of the *hDAB2IP* gene mediated by epigenetic control in PCa cells.

EXPERIMENTAL PROCEDURES

Cell Cultures and Treatments—Two human prostate cancer cell lines (LNCaP and PC-3) were maintained in T medium supplemented with 5% fetal bovine serum as previously described (2). Two normal human prostate cell lines (PrEC, a primary prostatic epithelial cell line derived from a 17-year-old juvenile prostate; and PZ-HPV-7, an immortalized cell line derived from the peripheral zone of a normal prostate) were maintained in a chemically defined medium (PrEGM) purchased from BioWhittaker, Inc. (Walkersville, MD).

Cells were seeded at low density (6×10^5 /100-mm dish) 16 h prior to treatment with different agents at the indicated final concentrations: 25, 100, or 200 nM trichostatin A (TSA; Sigma) or 1, 5, or 10 μ M 5'-aza-2'-deoxycytidine (5'-Aza; Sigma). For TSA treatment, medium containing fresh agent was changed every 24 h for 48 h. For 5'-Aza treatment, medium containing fresh agent was changed every 48 h for 96 h. For combined treatment, TSA was added at 24 h and changed at 72 h, and 5'-Aza was replaced at 48 h. Cells were collected at 96 h after treatment.

Real-time Reverse Transcription-PCR Assay—Total cellular RNA

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF367051.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains Supplemental Data and Supplemental Figs. 1 and 2.

¹ To whom correspondence should be addressed: Dept. of Urology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9110. Tel.: 214-648-3988; Fax: 214-648-8786; E-mail: JT.Hsieh@UTSouthwestern.edu.

¹ The abbreviations used are: PCa, prostate cancer; TSA, trichostatin A; 5'-Aza, 5'-aza-2'-deoxycytidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RLA, relative luciferase activity; ChIP, chromatin immunoprecipitation; PSA, prostate-specific antigen; HDAC, histone deacetylase.

TABLE I
PCR primers used on bisulfite-treated DNA and in ChIP assays

R = A or G; Y = C or T.

PCR target	Primer name	Primer 5'-3' sequence	Target size bp	Genomic positions
Bisulfite-treated DNA	F-PmIIa	GGATTTTCTTAGGTGGGTGT	236	-522/-503
	R-PmIIa	CCCTAAACCRCTATTACCTTAAC	236	-285/-308
	F-PmIIb	GTTAAGGTAATAGYGGTTAGGG	397	-308/-285
	R-PmIIb	ACRAACTCACCTCTCATTATCC	397	+89/+68
	F-PmI	GGTGAGTAGAAGAGAGAGAGTA	694	+282/+304
	R-PmI	CCTAAACCCCTAAAACTATATCC	694	+975/+952
	F-PmII (outer)	CGCTCTGGGAAGGAAAGTCG	419	-704/-685
	F-PmII (inner)	ATTCTCTCCAGGTGGGTGGG	233	-520/-501
Immunoprecipitated DNA (ChIP)	R-PmII (inner)	CTAAGCCGCTGTTGCCTTGGC	233	-287/-308
	R-PmII (outer)	CCTAAGCCGCTGTTGCCTTG	419	-286/-306
	F-PI (outer)	GGGGAAGGCTGAACATCTGG	486	+539/+558
	F-PI (inner)	CTGCTTCTCTTTCTCTCTCTG	105	+768/+791
	R-PI (inner)	TGAACACCTCCTCCTCCTCTC	105	+873/+850
	R-PI (outer)	GCCTGTGCCTAAGTGGAAGG	486	+1025/+1005

was isolated from PC-3, LNCaP, and PZ-HPV-7 cells using RNazol B (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. Two micrograms of total cellular RNA were used in each reaction. cDNA was synthesized and amplified using either the *hDAB2IP* primer set (2 ng/ μ l) (F-*hDAB2IP*, 5'-TGGACGATGTGCTC-TATGCC-3'; and R-*hDAB2IP*, 5'-GGATGGTGATGTTGGTAG-3') or the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set (6 ng/ μ l) (G3P7, 5'-GAAGGTGGGTCCGAGTCAACG-3'; and G3P4, 5'-AGTGAGCTTCCCGTTCAGC-3') in a 40- μ l reaction mixture containing 20 μ l of platinum qPCR Supermix-UDG (Invitrogen) and 4 μ l of SYBR Green I (final dilution of 1:10,000). The reactions were carried out on a 96-well plate, and a PCR amplification protocol was followed (95 °C for 3 min and 40 cycles of amplification at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min) using an iCycler iQ machine (Bio-Rad). A quality control was carried out using both electrophoresis analysis on a 2% NuSieve agarose gel (3:1; FMC Corp. BioProducts) and melting curve analysis performed immediately after the end of amplification at 95 °C for 1 min and 55 °C for 1 min and 80 cycles of 0.5 °C increments beginning at 55 °C. We also performed the standard curves for *hDAB2IP* and GAPDH to ensure the linearity and efficiency of both genes. The linear range of both genes is from 2×10^2 to 2×10^6 copies, and the efficiency of each reaction ranges from 92 to 97%. The relative induction of *hDAB2IP* mRNA was calculated as follows: ΔC_t (threshold cycle) of each sample = mean of $C_{t(hDAB2IP)}$ - mean of $C_{t(GAPDH)}$. The fold induction of each sample = $1/2^{\Delta C_t(\text{sample}) - \Delta C_t(\text{control})}$.

Construction of Reporter Gene Vectors—To analyze the promoter region of *hDAB2IP*, a 7.6-kb fragment from bacterial artificial chromosome (BAC) clone 298A17 (GenBank™/EBI accession number AL365274) containing the predicted first exon and additional 5'-upstream sequence of the *DAB2IP* gene was subcloned into the *EcoRI* site of pBluescript SK(-) (Stratagene) (1). pGL3-1.6S, a 1.6-kb *KpnI-XhoI* fragment, was subcloned from this 7.6-kb element into the pGL3-Basic vector (Promega). Two putative promoter regions (P1, a 0.8-kb *SfiI-XhoI* fragment from +229 to +981; and P2, a 0.6-kb *KpnI-Kpn2I* fragment from -598 to +44) were subcloned into the pGL3-Basic vector (see Fig. 1A).

To further analyze the regulation of *hDAB2IP* promoters, two sets of primers (F-PI (inner, 5'-CCTGCTTCTGTTCTCTCTCTG-3') and R-PI (inner, 5'-TTGAACACCTCCTCCTCTCTC-3'); F-PmII (inner, 5'-ATTCTCTCCAGGTGGGTGGG-3') and R-PmII (inner, 5'-CCTAAGC-CGCTGTTGCTTG-3')) were used to amplify the PI (+768 to +873) and PmII (-520 to -287) fragments. PCR fragments were cloned into pCR2.1-TOPO (Invitrogen), sequenced, and then subcloned into the pGL3-Basic vector using *HindIII-XhoI* sites.

Cell Transfection and Luciferase Reporter Assay—We plated cells at a density of 1.0×10^5 cells/well on a six-well plate. After 16 h, we transfected the PZ-HPV-7 and PC-3 cell lines with both 0.8 μ g of reporter vectors and 0.2 μ g of β -galactosidase vector (pCH110) using FuGENE 6 (Roche Molecular Biochemicals). The LNCaP and PrEC cells were transfected with the same amount of DNA with LipofectAMINE Plus transfection reagent (Invitrogen). Twenty-four hours after incubation, the transfected cells were treated with TSA for 24 h, 5'-Aza for 48 h, or a combination of both drugs by incubating with 5'-Aza for 24 h and then adding TSA for an additional 24 h. After washing twice with cold phosphate-buffered saline, the cells were harvested with lysis buffer (Promega). Both luciferase and β -galactosidase

activities were assayed as previously described (1, 12). The protein concentration of each extract was measured using the Bio-Rad protein assay. The relative luciferase activity (RLA) was calculated by normalizing both β -galactosidase and protein concentrations in each sample, and the data were averaged from RLA in triplicate.

Acid Extraction of Histone and Western Analysis—Cells were scraped, centrifuged at $200 \times g$ for 10 min, and then suspended in 10 volumes of phosphate-buffered saline. Cells were spun down; pellets were suspended in 5 volumes of lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 1.5 mM phenylmethylsulfonyl fluoride); and sulfuric acid was added to a final concentration of 0.2 M, followed by incubation on ice for 30 min. After centrifugation at $11,000 \times g$ for 10 min at 4 °C, the cell supernatant containing the acid-soluble fraction was retained. The supernatant was dialyzed twice against 200 ml of 0.1 M acetic acid for 1–2 h and then dialyzed overnight against 200 ml of H_2O using Spectrapor® molecular porous membrane tubing (Spectrum Medical Industries, Inc., Los Angeles, CA). The protein concentration was measured, and proteins were fractionated by SDS-PAGE (15%). Western blotting was carried out with an anti-acetylhistone H3 antibody (1:3000; Upstate Biotechnology, Inc., Lake Placid, NY). The same membrane was stripped and reprobed with an anti-histone H3 antibody (1:1000; Upstate Biotechnology, Inc.).

Chromatin Immunoprecipitation (ChIP) Assay—After treatment, formaldehyde was added to the cell medium at a final concentration of 1% for cross-linking proteins to DNA. Cells were washed, scraped off with ice-cold phosphate-buffered saline, and resuspended in SDS lysis buffer containing a mixture of protease inhibitors. An equal protein concentration of cell lysate from each sample was sonicated to reduce DNA fragments between 200 and 1000 bp. Once the cell debris was removed, the supernatant was diluted in ChIP dilution buffer (1:10), and 1% of this supernatant (as input DNA) was collected, purified, and subjected to genomic PCR with the primer sets described in Table I. Samples were precleared with salmon sperm DNA/protein A-agarose slurry (Upstate Biotechnology, Inc.) and incubated overnight at 4 °C with or without (as a negative control) antibody. Immune complexes were collected by adding salmon sperm DNA/protein A-agarose slurry and incubated with 20 μ l of 5 M NaCl at 65 °C to reverse DNA-protein cross-linking. DNA was then purified by proteinase K digestion, phenol extraction, and ethanol precipitation.

The strand-specific nested PCR primers used for amplifying the *hDAB2IP* gene are indicated in Table I. PCR amplifications were performed in a 50- μ l reaction mixture containing 2 μ l of DNA by the addition of 2 units of ThermalAce™ DNA polymerase (Invitrogen). A hot start was performed (98 °C for 3 min), followed by 30 cycles at 98 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s. The PCR product from the PI region (~110 bp) was separated on 4% E-Gel™ (Invitrogen), and that from the PmII region (~230 bp) was separated on a 2% NuSieve agarose gel (3:1).

Bisulfite Genomic Sequencing—High molecular mass genomic DNA was obtained from PrEC, PZ-HPV-7, LNCaP, and PC-3 cell lines and subjected to bisulfite modification (13, 14). Briefly, 1–2 μ g (5–10 μ l) of genomic DNA were denatured with NaOH (final concentration of 0.2 M), 30 μ l of 10 mM hydroquinone (Sigma), and 520 μ l of 3 M sodium bisulfite (Sigma) at pH 5 for 16 h at 50 °C. The modified samples were purified

using Wizard DNA Clean-Up system desalting columns (Promega), followed by ethanol precipitation. Bisulfite-modified DNA (100 ng) was amplified by PCR in a 25- μ l reaction mixture containing the primers indicated in Table I. A hot start was performed (95 °C for 5 min) by adding 0.5 unit of HotStar Taq DNA polymerase (QIAGEN Inc., Valencia, CA). The PCR products were cloned into the TA cloning vector pCR2.1-TOPO. Four to eight individual clones were sequenced using reverse and forward M13 primers.

RESULTS

Identification of Two *hDAB2IP* Promoters in Prostatic Epithelial Cell Lines—Two putative promoters, P1 (+229 to +981, located within the first intron) and P2 (−598 to +44, located 5′-upstream of exon 1a) (Fig. 1A), were identified using both the TSSW program (human PII recognition using the TRANSFAC Database)² and experimental deletion analysis (1) of a 1662-bp *hDAB2IP* locus surrounding the transcription initiation site (+1). As shown in Fig. 1B, there are many GC-rich sequences, and potential transcription factor-binding sites were detected within this region using MacVector Version 6.5.3.

To analyze the basal activity of each *hDAB2IP* promoter in various prostate cells, luciferase reporter vector constructs were generated. Using pGL3-1.6S, we detected the highest luciferase activity in both PrEC and PZ-HPV-7 cells, an intermediate level in LNCaP cells, and the lowest level in PC-3 cells (Fig. 1C), correlating with the steady-state levels of *hDAB2IP* mRNA in each cell line (1). Similar patterns of reporter gene activity were detected in these four cell lines using either the P1 or P2 promoter (Fig. 1C).

Induction of *hDAB2IP* Gene Expression by a Hypomethylation Agent (5′-Aza) and/or a Histone Deacetylase Inhibitor (TSA)—Apparently, the decreased *hDAB2IP* mRNA levels detected in many human PCa cells (1) could be caused by its reduced gene promoter activity (Fig. 1C). To understand the mechanism leading to the down-regulation of the *hDAB2IP* gene in human PCa cells, we first examined the role of epigenetic regulation of the *hDAB2IP* gene. The data from a ChIP assay demonstrated that the presence of acetylhistone H3 was associated with the P1 and PII regions of *hDAB2IP* in both PrEC and PZ-HPV-7 cells, but not in PC-3 and LNCaP cells (Fig. 1D), suggesting that histone acetylation may play a role in modulating *hDAB2IP* gene expression. Recent data also indicate that epigenetic controls such as histone acetylation and/or DNA methylation play cooperative roles in modulating gene expression, particularly genes involved in tumor suppression (15, 16). We therefore treated these cells with TSA, 5′-Aza, or a combination of both. The levels of *hDAB2IP* mRNA expression were evaluated by real-time reverse transcriptase-PCR using GAPDH as an internal control. As shown in Table II, TSA and/or 5′-Aza failed to elicit any elevation of *hDAB2IP* mRNA because the basal activity of the *hDAB2IP* promoter was very high in PZ-HPV-7 cells (Fig. 1C). However, in PC-3 cells (Table II), either TSA or 5′-Aza was able to induce *hDAB2IP* mRNA expression. In contrast, the increased *hDAB2IP* mRNA levels in LNCaP cells treated with a single agent were lower than those in PC-3 cells (Table II) because LNCaP cells had higher endogenous *hDAB2IP* mRNA levels compared with PC-3 cells (1). For the combination of both agents, the level of induction exhibited an additive effect only in the PC-3 and LNCaP cell lines. In some cases, we noticed that the mRNA levels after the combination treatment were lower than those after the single-agent treatment, which was caused by the toxicity of the drug combination.

Characterization of the *hDAB2IP* Promoter Regulated by Histone Acetylation and DNA Methylation—To delineate which promoter could be induced by TSA, 5′-Aza, or a combination of

both drugs and the underlying mechanism of the induction, we transiently transfected PCa cells with pGL3-P1 or pGL3-P2 under the same treatment conditions. In PC-3 cells, TSA could induce P1 promoter activity in a dose-dependent manner; however, 5′-Aza only slightly induced this promoter activity (Fig. 2A). The combination treatment exhibited an additive effect only on P1 activity. In contrast, a very different induction pattern was observed in LNCaP cells transfected with pGL3-P1; only marginal induction of P1 activity was observed in these cells after the different treatments (Fig. 2B).

By transfecting pGL3-P2 into PC-3 cells, a dose-dependent induction pattern of P2 activity was observed in these cells treated with either TSA or 5′-Aza (Fig. 2C). In LNCaP cells, P2 activity could also be induced by either TSA or 5′-Aza in a dose-dependent manner (Fig. 2D), which differed from P1 activity induced by these drugs (Fig. 2B). Again, in PC-3 and LNCaP cells, the combined treatment with TSA and 5′-Aza exhibited an additive effect only on P2 activity. In addition, we determined both P1 and P2 activities in PC-3 and LNCaP cells with a different transfection protocol; the overall induction pattern was consistent (see Fig. 1 in the Supplemental Material). Taken together, these results indicate that the P2 promoter is responsible for both TSA- and 5′-Aza-induced *hDAB2IP* gene expression in PC-3 and LNCaP cell lines.

To evaluate the possibility of a global gene induction effect of TSA or 5′-Aza on PCa cells, we examined the activity of the prostate-specific antigen (PSA) gene promoter in LNCaP and PC-3 cells treated with either agent. As shown in Fig. 2E, no induction of PSA reporter activity was detected in both cell lines treated with a single agent or a combination of both agents. In contrast, androgen could induce PSA reporter activity dramatically in LNCaP cells (androgen receptor-positive), but not in PC-3 cells (androgen receptor-negative). Therefore, we believe that TSA or 5′-Aza has a specific effect on regulating *hDAB2IP* gene expression in PCa cell lines.

Increased Levels of Acetylhistone H3 in the *hDAB2IP* Promoter Induced by TSA—To determine whether the TSA-induced *hDAB2IP* gene expression correlated with the levels of histone acetylation associated with the *hDAB2IP* promoter region, we analyzed the steady-state levels of acetylhistone H3 in both PC-3 and LNCaP cells after TSA treatment. As shown in Fig. 3A, Western blot analysis of PC-3 cells indicated that the basal level of acetylhistone H3 was very low, whereas TSA induced a dramatic elevation of the ratio between acetylhistone H3 and total histone H3. Comparing this with the no-treatment control, TSA induced a dose-dependent (ranging from 8- to 88-fold) elevation of acetylhistone H3. In contrast, the basal level of acetylhistone H3 was very high in LNCaP cells (Fig. 3B). Therefore, we failed to detect any changes in the steady-state levels of acetylhistone H3 in LNCaP cells treated with TSA. Nevertheless, it is still possible that TSA increases the acetylhistone H3 levels associated with the *hDAB2IP* promoter region.

To analyze the status of acetylhistone associated with the *hDAB2IP* promoter, a ChIP assay was performed using the sequences corresponding to the P1 (+768 to +873) and PII (−520 to −287) regions (Fig. 1A). Elevated levels of acetylhistone H3 were clearly associated with the P1 region in both PC-3 and LNCaP cells treated with TSA or the combination, but not with 5′-Aza (Fig. 4A). Similarly, an accumulation of acetylhistone H3 levels associated with the PII region was also detected in both cell lines treated with TSA or the combination (Fig. 4B). Interestingly, we also found that 5′-Aza treatment could induce the accumulation of acetylhistone H3 in the PII region, but not in the P1 region (Fig. 4B), because P2 (but not P1) activity could be induced in both PCa cell lines treated with 5′-Aza (Fig. 2). A

² Available at genomic.sanger.ac.uk/gf/gf.htm.

TABLE II

Determination of *hDAB2IP* mRNA expression induced by TSA and 5'-Aza by real-time reverse transcriptase-PCR

Each data point was averaged from two different experiments performed in duplicate using real-time reverse transcriptase-PCR. After calculating the mean $\pm$ S.D. of *C_t* for each sample, the S.D. of *hDAB2IP* from all data was <5% of its mean, and the S.D. of GAPDH from all data was <9% of its mean. The fold induction was calculated as described under "Experimental Procedures" using the control (taken as 1) of each cell line. ND, not determined.

Combination	Cell line		
	PZ-HPV-7	PC-3	LNCaP
	-fold		
TSA (nM)/5'-Aza (μ M)			
0/0	1.00	1.00	1.00
25/0	0.83	4.02	1.88
100/0	0.80	5.05	2.42
200/0	0.83	8.31	2.44
0/1	0.83	3.82	3.58
0/5	0.92	4.03	4.47
0/10	1.08	4.20	ND
25/1	1.07	8.02	5.63
100/5	0.97	9.29	6.83
200/10	ND	5.34	ND

gated the luciferase activity of two constructs, pGL3-P1 (+768 to +873) and pGL3-P11 (-520 to -287), in PC-3 and LNCaP cell lines after treatment. As shown in Fig. 5 (A and B), the basal luciferase activity of the pGL3-P1 construct was much higher than that of the pGL3-P11 construct in both PC-3 and LNCaP cells (Fig. 2, A and B), suggesting that the deletion of 5'- and 3'-flanking sequences from the P1 region may contain some negative elements. Overall, we detected a slight increase in P1 activity only in PC-3 cells treated with TSA or a combination of both TSA and 5'-Aza (Fig. 5A); however, no change in P1 activity was detected in LNCaP cells after treatment (Fig. 5B).

We observed a dramatic induction of PII activity in PC-3 cells after treatment (10–18-fold increase with TSA and 14–30-fold increase with the combination) (Fig. 5C). Using 5'-Aza, an ~3-fold induction of PII activity was detected in PC-3 cells. A similar induction profile of PII activity was detected in LNCaP cells (Fig. 5D). For example, TSA alone induced an ~6–14-fold increase in PII activity, whereas the combination treatment induced an 8–44-fold increase in reporter gene activity. An ~2-fold induction of PII activity was observed in LNCaP cells treated with 5 μ M 5'-Aza. In addition, we repeated these experiments with a different transfection protocol; the overall induction pattern was consistent (see Fig. 2 in the Supplemental Material). Taken together, these data suggest that PII (-520 to -287) within the *hDAB2IP* promoter is the core regulatory region for modulating *hDAB2IP* gene transcription.

Characterization of the Methylation Status of the *hDAB2IP* Promoters in Prostate Cell Lines—It is known that aberrant methylation (which is associated with gene silencing) in the promoters of tumor suppressor genes is commonly detected in cancer cells (18–20). CpG islands appear to be critical sites modulated by DNA methylation (21–23). Because the 5'-regulatory region in the *hDAB2IP* promoter is GC-rich and the DNA hypomethylation agent (5'-Aza) can induce *hDAB2IP* gene expression, determining the methylation profile of the promoter region in normal and cancerous cells could provide additional evidence for the role of DNA methylation in the regulation of *hDAB2IP* during PCa development. In this experiment, two PCa cell lines (PC-3 and LNCaP) and two normal prostate cell lines (PrEC and PZ-HPV-7) were subjected to bisulfite genomic sequencing. With respect to the high GC content in the *hDAB2IP* promoter, primers were designed to avoid potential methylation sites (e.g. CpG) such that both methylated and un-

methylated DNAs would be amplified equally. For the P1 region, PmI (spanning 35 CpG sites) was designed; and for the P2 region, PmIIa (spanning 30 CpG sites) and PmIIb (spanning 56 CpG sites) were designed (Fig. 6A) because we found more CpG sites in the P2 region (86 sites) than in the P1 region (35 sites). The detailed primer information is summarized in Table I.

In the PmI region, PC-3 cells showed a partial methylation pattern, and LNCaP cells showed an almost completed methylation pattern. In contrast, PZ-HPV-7 cells showed a completed unmethylation pattern, and PrEC cells contained very few methylation sites (Fig. 6B). The density of methylation of this region correlated inversely with the basal activity of the P1 promoter in all cells examined (Fig. 1C).

In the PmIIa region, both normal prostate cell lines showed an almost completed unmethylated pattern. However, LNCaP cells contained low densities of methylation, whereas PC-3 showed a significantly higher degree of methylation pattern (Fig. 6C). This evidence indicated that methylation density in the PmIIa region inversely correlated with the basal activity of P2 in these cells (Fig. 1C). Interestingly, in the PmIIb region, PC-3, PZ-HPV-7, and PrEC cells showed almost completed unmethylated patterns, and LNCaP cells contained few methylation sites (data not shown). Taken together, these data clearly indicate that the PmIIa region (-522 to -285) in *hDAB2IP* is the key regulatory sequence operative in prostatic epithelia.

DISCUSSION

The higher levels of *hDAB2IP* mRNA detected in normal prostatic epithelia compared with PCa cells are mainly regulated at the transcriptional level (1). In this study, we further demonstrated that the activity of the *hDAB2IP* promoter is more active in normal prostatic epithelia than in PCa cells (Fig. 1C). We also noticed that the 5'-upstream regulatory region of the *hDAB2IP* gene has GC-rich sequences, but no canonical TATA boxes. This is a typical feature of the promoters of many housekeeping genes and of ~40% of tissue-specific genes (24). Although various mechanisms may underlie this repression in PCa cells, our data demonstrate that histone acetylation and/or DNA methylation plays a crucial role in modulating *hDAB2IP* gene expression in PCa cells. The treatment of PCa cell lines such as PC-3 and LNCaP with TSA, 5'-Aza, or a combination of both significantly increased the steady-state levels of *hDAB2IP* mRNA (Table II). In contrast, TSA or 5'-Aza could not induce *hDAB2IP* gene expression in normal prostatic epithelia (Table II). These data indicate that both DNA methylation and histone deacetylation act cooperatively to silence the *hDAB2IP* gene in PCa cells. Such action is presumably mediated through a complex chromatin structure in which methyl-CpG-binding proteins are associated with histone deacetylases (HDACs) (25, 26).

Eukaryotic DNA is packed into a highly organized structure (27). It has become increasingly clear that gene transcription from this tightly packed DNA is regulated by chromatin-re-modeling events, which can render DNA either more or less accessible to transcription factors. One of the key events in the regulation of eukaryotic gene expression is the post-translational modification of nucleosomal histones, which convert regions of chromosomes to transcriptionally active or inactive. The most well studied post-translational modification of histones is the acetylation of ϵ -amino groups on positively charged lysine residues in histone amino-terminal tail domains (7, 28), which can release negatively charged DNA to interact with transcription factors. The effect of histone acetyltransferases (29) is counterbalanced by the presence of (HDACs) (30). Aberrant acetylation or deacetylation leads to such diverse disorders as leukemia, epithelial cancers, fragile X syndrome, and

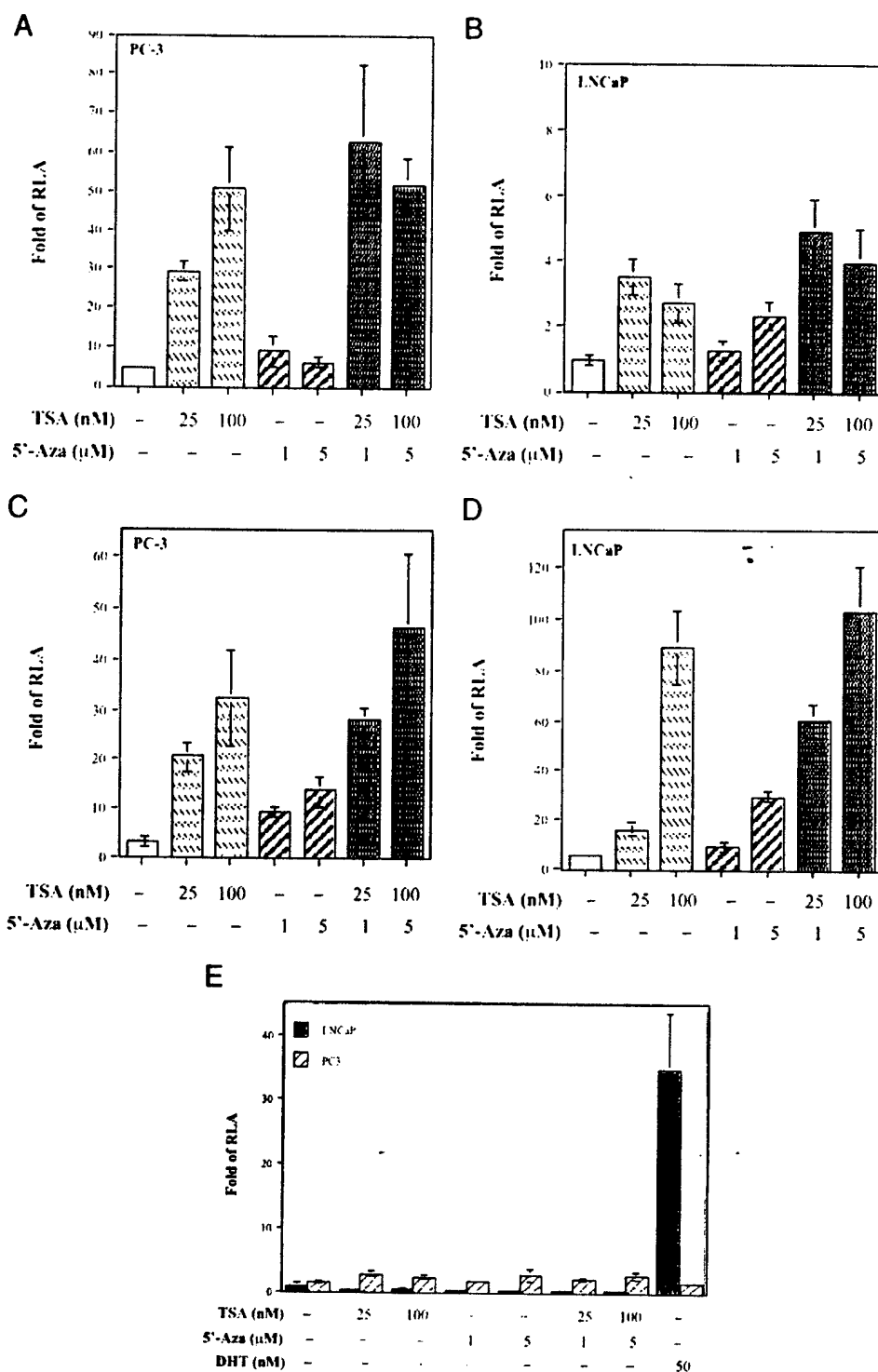


FIG. 2. Effect of TSA and/or 5'-Aza on *hDAB2IP* or PSA promoter activity in PCa cell lines. Either PC-3 or LNCaP cells were transfected with pGL3-P1 (A and B), pGL3-P2 (C and D), or pPSA6.1 (E) under different treatment conditions, and luciferase activity was determined as described under "Experimental Procedures." The -fold RLA was calculated using the pGL3-Basic vector (taken as 1). DHT, dihydrotestosterone.

Rubinstein-Taybi syndrome (31). It is also known that HDACs can function as transcriptional corepressors and are often present in multisubunit complexes such as Sin3 and Mi2 complexes (32–34). From recent reports, HDAC-containing complexes are involved in DNA methylation-mediated transcriptional silencing of various tumor suppressor genes (15, 16). Therefore, targeting HDAC activity has become a new strategy of cancer chemotherapy; several inhibitors have been developed and tested in clinical trials (35). Recent studies by several groups (36–38) have demonstrated the existence of cellular complexes

containing both HDACs and ATP-dependent nucleosome-remodeling activity, suggesting that some chromatin remodeling is mediated by cellular complexes with HDAC activity. In contrast, histone acetyltransferases such as CBP (cAMP-responsive element-binding protein)/p300, CBP-associated factor (PCAF), and GCN5 have been identified in the protein complex of transcriptional activators (39–41).

DNA hypermethylation has been implicated in parental gene imprinting, X chromosome inactivation, and endogenous retrovirus silencing (42–46) as well as in the transcriptional silenc-

FIG. 3. Steady-state levels of histone H3 acetylation in PCa cells induced by TSA. After TSA treatment, the acid extract of nuclear protein from PC-3 cells (A) or LNCaP cells (B) was subjected to Western blot analysis and probed with an anti-acetylhistone H3 antibody (1:3000). The same membrane was stripped and reprobed with an anti-histone H3 antibody (1:1000) as an internal control. The values depicted beneath each lane represent the relative levels of acetylhistone H3 determined by normalizing the amount of acetylhistone H3 proteins to that of total histone H3 proteins.

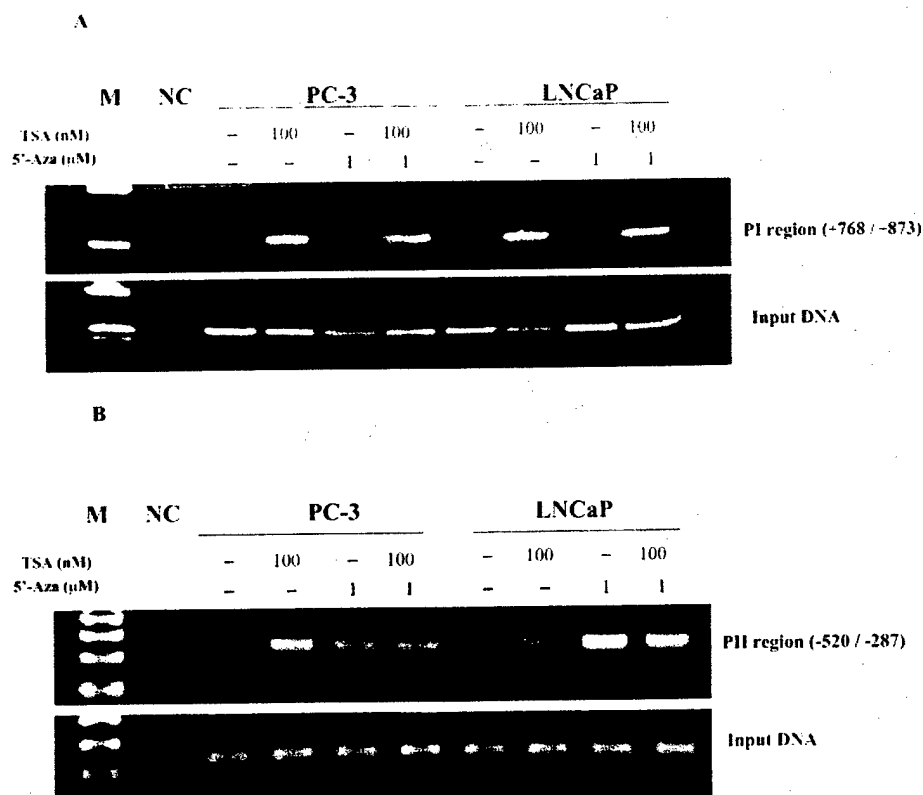
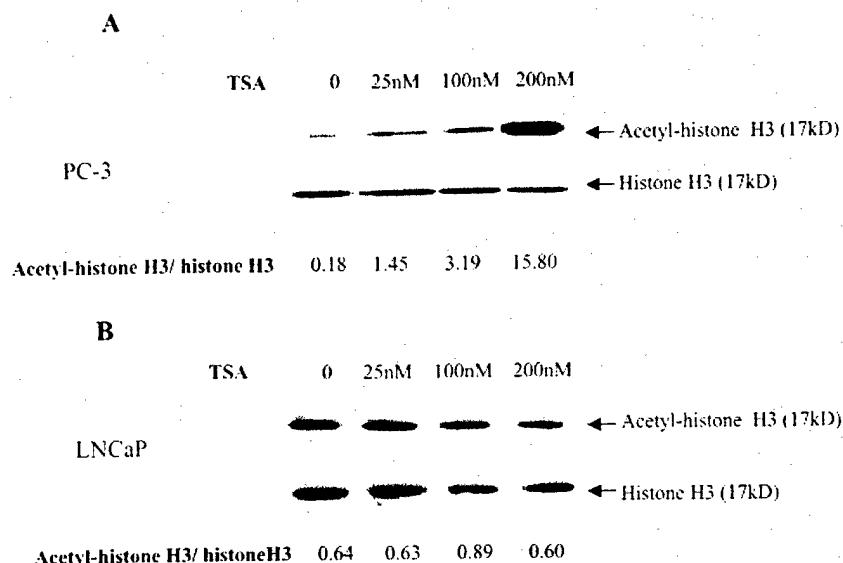


FIG. 4. Increased levels of histone H3 acetylation associated with the *hDAB2IP* promoter in PCa cells treated with TSA and/or 5'-Aza. The ChIP assay was performed using an anti-acetylhistone H3 antibody. Nested PCR to detect the PI (A) and PII (B) regions was performed using the primer sets summarized in Table I. The input DNA (lower panels) was used as a positive control. *M*, 1-kb plus marker; *NC*, negative control without antibody.

ing of tumor suppressor genes (47, 48). Hypermethylation of CpG islands is also found in the 3'-ends of some genes; however, the density of DNA methylation in promoter or first exon regions correlates inversely with gene transcription (22, 49). It has also been shown that transcription repression mediated by methyl-CpG-binding proteins involves an HDAC complex (50, 51), indicating that there is a close relationship between DNA methylation and histone deacetylation.

Regarding the potential role of histone acetylation, data from the ChIP assays indicated that acetylhistone H3 was associated with the *hDAB2IP* promoter in normal epithelial cell lines (PrEC and PZ-HPV-7) expressing *hDAB2IP* proteins (Fig. 1D). A dramatic increase in the levels of acetylhistone H3 associated with the *hDAB2IP* promoter was detected in PCa cells in the presence of TSA (Fig. 4, A and B). We further demonstrated

that the DNA fragment identified in the ChIP assay had promoter activity and could respond to TSA treatment (Fig. 5, C and D). Based on these results, we conclude that the status of acetylhistone in the *hDAB2IP* promoter is critical for its gene regulation. We also noticed that several potential transcription factor-binding sites such as AP-1, AP-2, interferon-stimulated response element, CCAAT box-binding transcription factor-nuclear factor 1 (CTF-NF1), and adenovirus early region 4 promoter transcription factor 1 (E4TF1) and a cluster of Sp1-binding sites located in this region (Fig. 1B). In particular, members of the Sp1 family have been shown to act as positive or negative regulators of gene transcription. This mechanism is dependent on the competition between the transcription repressor HDAC1 and the transcription factor E2F1, which activates histone acetyltransferase (52). The presence of Sp1-bind-

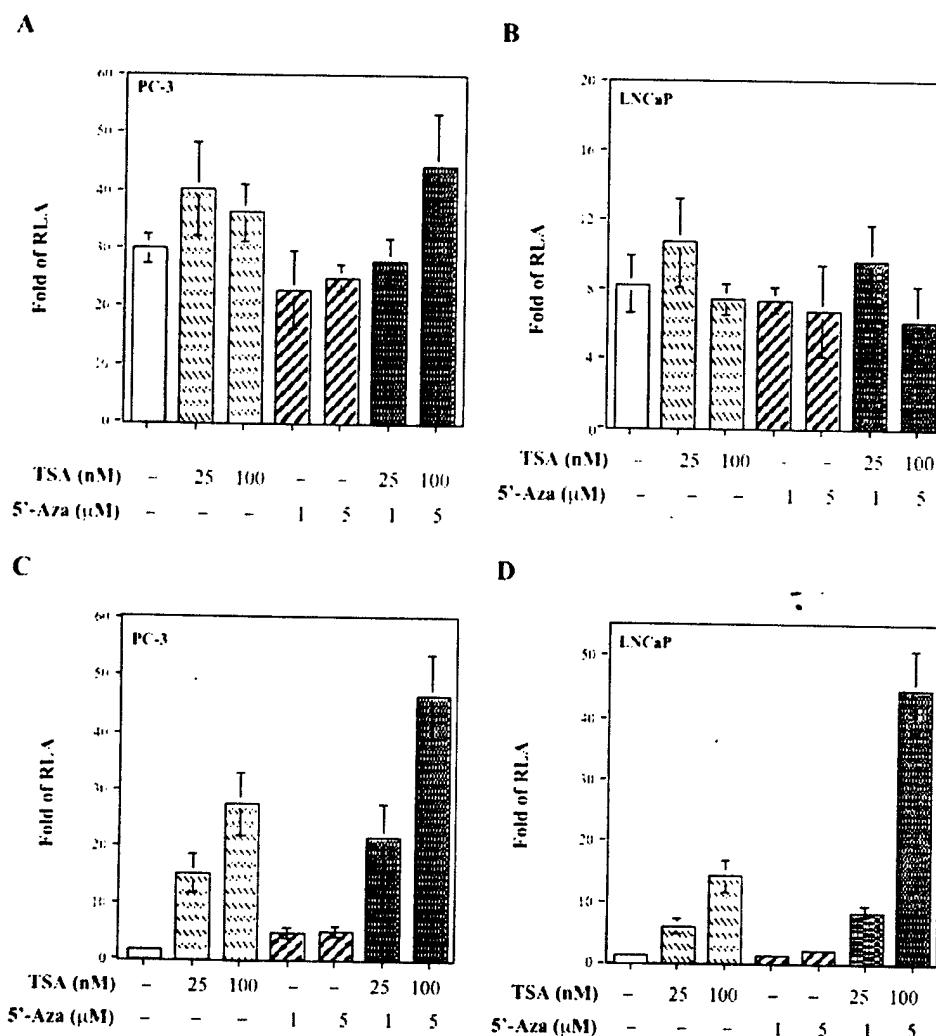


FIG. 5. Characterization of the core promoter in the *hDAB2IP* gene regulated by TSA and/or 5'-Aza. Constructs pGL3-P1 (A and B) and pGL3-P2 (C and D) derived from a ChIP assay were transfected into PC-3 or LNCaP cells under different treatment conditions, and RLA was determined as described under "Experimental Procedures." The -fold RLA was calculated using the pGL3-Basic vector (taken as 1).

ing elements in the proximal *hDAB2IP* gene promoter could underlie the basis of gene repression mediated by histone deacetylation (53, 54). Further investigation is warranted.

Regarding the role of DNA methylation in regulating *hDAB2IP* gene transcription, bisulfite sequencing data indicated that CpG islands remained almost unmethylated in normal prostate cell lines (PrEC and PZ-HPV-7) expressing the transcriptionally active *hDAB2IP* gene (Fig. 1C) (1). However, in PCa cells (PC-3 and LNCaP), hypermethylation of CpG islands was commonly associated with the *hDAB2IP* promoter region (Fig. 6). 5'-Aza could induce the expression of *hDAB2IP* mRNA in PCa cells (Table II). Our results are consistent with the promoter activity determined by the reporter gene assay (Fig. 2).

Regarding the regulation of the *hDAB2IP* gene, our data clearly demonstrate that DNA methylation and histone deacetylation can act cooperatively in silencing the *hDAB2IP* gene (Table II and Fig. 4). It has been shown that DNA methyltransferases recruited by an oncogene to a gene promoter suppress the expression of this gene (55). Also, the binding of the methyl-CpG-binding protein complex (21) to methyl-CpG islands competes with transcription factors and prevents them from binding to the promoter region. Recent data indicate that the methyl-CpG-binding protein can recruit HDACs, leading to condensation of the local chromatin structure and thereby ren-

dering the methylated DNA less accessible to transcription factors (25).

In the *hDAB2IP* gene, there are two regions with potential promoter activity: P1 and P2. In this study, we found that P2 promoter activity has a better correlation with the induction of *hDAB2IP* mRNA in every tested cell line (Fig. 1C). The methylation profile of the P2 promoter in each cell line exhibited a reciprocal relationship between P2 reporter gene activity (Fig. 5C) and the density of methylated cytosine residues (Fig. 6C). Furthermore, the P2 (but not P1) promoter was able to respond to both TSA and 5'-Aza treatment (Fig. 2). Nevertheless, TSA seemed more potent than 5'-Aza in eliciting P2 promoter activity (Figs. 2 and 5). Therefore, we believe that the P2 region in the *hDAB2IP* gene represents a core promoter in prostatic epithelia. Our results also suggest that the deacetylhistone-mediated transcriptional silencing of the *hDAB2IP* gene may be a critical event during the carcinogenesis of PCa.

In summary, cytosine methylation and histone deacetylation in the *hDAB2IP* regulatory regions associated with the silencing of *hDAB2IP* gene expression have been observed in PCa cells (PC-3 and LNCaP). Such a phenomenon seems to be specific to cancer because it was not detected in normal prostate cells (PZ-HPV-7 and PrEC). Therefore, this gene could potentially serve as a surrogate marker for early cancer detection. The outcome of this study also indicates that histone

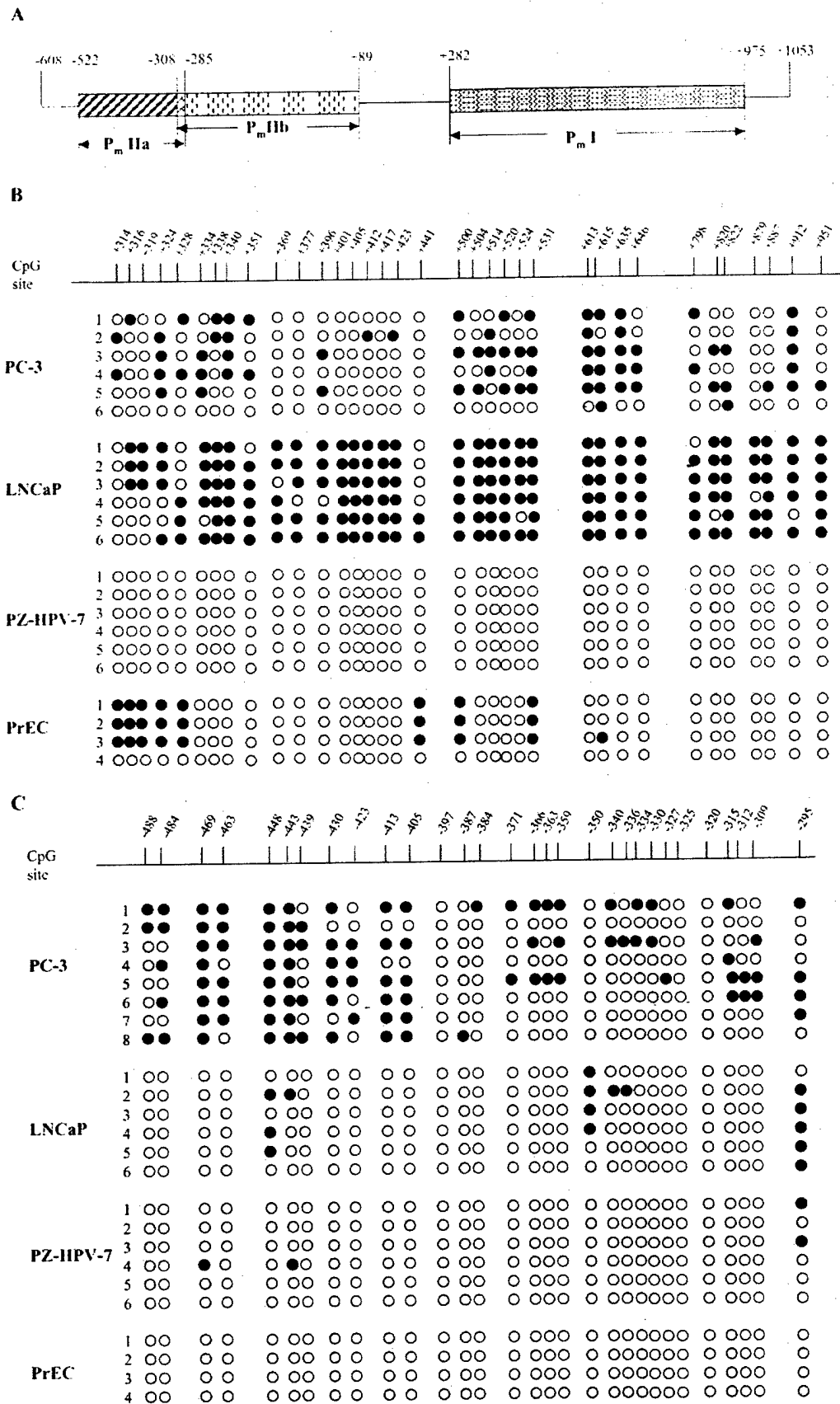


FIG. 6. Characterization of the methylation status of the *hDAB2IP* gene promoter in human prostatic epithelial cells. A, schematic representation of the three separated positions in the *hDAB2IP* locus subjected to bisulfite sequencing analysis. B and C, methylation patterns in the PmI (+282 to +975) and PmIIa (-522 to -285) regions in human prostatic epithelial cells. High molecular mass DNA isolated from each sample was modified with sodium bisulfite and amplified by PCR using the primer sets indicated in Table I. The PCR product was subcloned, and each individual clone (horizontal rows) from every sample was sequenced. The position of each CpG dinucleotide (vertical bars) is labeled with the number representing its location in the *hDAB2IP* gene. ○, unmethylated CpG; ●, methylated CpG.

deacetylase and DNA methyltransferase can be novel targets for PCa therapy.

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Characterization of a Novel Negative Regulator (DOC-2/DAB2) of c-Src in Normal Prostatic Epithelium and Cancer*

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Jian Zhou, Jessica Scholes, and Jer-Tsong Hsieh†

From the Department of Urology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9110

DOC-2/DAB2 is a potent tumor suppressor in many cancer types including prostate cancer. In prostate cancer, expression of DOC-2/DAB2 can inhibit its growth. Our recent studies demonstrate that DOC-2/DAB2 can suppress both protein kinase C and peptide growth factor-elicited signal pathways via the Ras-mitogen-activated protein kinase pathway. In this study, we further showed that the proline-rich domain of DOC-2/DAB2 could also interact with proteins containing the Src homology 3 domain, such as Src and Fgr. The binding of c-Src to DOC-2/DAB2 was enhanced in cells treated with growth factor, and this interaction resulted in c-Src inactivation. The c-Src inactivation was evidenced by the decreased tyrosine 416 phosphorylation of c-Src and reduced downstream effector activation. It appears that DOC-2/DAB2 can bind to Src homology 3 domain of c-Src and maintain it in an inactive conformation. Thus, this study provides a new mechanism for modulating c-Src in prostatic epithelium and cancer.

DOC-2/DAB2 (differentially expressed in ovarian carcinoma-2/disabled-2) is a gene that encodes a novel phosphoprotein involved in signal transduction (1–7). The aberrant expression of DOC-2/DAB2 is often in tumors such as ovarian, prostate, choriocarcinoma, and mammary tumors (1, 3, 8–10). Increased expression of DOC-2/DAB2 inhibits the growth of these tumors, indicating that DOC-2/DAB2 must play a key role in controlling growth-related signal pathways.

The phosphorylation of DOC-2/DAB2 can be induced by several stimuli, such as growth factors and protein kinase C activator-TPA.¹ We demonstrate that the serine 24 phosphorylation in the N terminus of DOC-2/DAB2 is required for its inhibitory effect on TPA-induced gene transcription (5). Using yeast two-hybrid system, we further identify an interactive protein (*i.e.* DIP1/2 or DAB2IP) associated with the N terminus of DOC-2/DAB2 protein (7). DIP1/2 protein is a new member of the Ras-GAP family, and its activity can be enhanced by interacting with DOC-2/DAB2 in prostate cancer (PCa) cells treated with TPA (7), which results in inhibiting TPA-induced gene transcription and cell growth. Therefore, we conclude that

DIP1/2 is a key downstream effector in DOC-2/DAB2-mediated signal cascade.

In addition, the C terminus of DOC-2/DAB2 contains unique motifs such as three proline-rich domains (*i.e.* amino acid 619–627, 663–671, and 714–722). We demonstrated recently (6) that one of these proline-rich domains (amino acid 663–671) can interact with Grb2, leading to the inhibition of both epidermal growth factor (EGF)- and neurotrophin (NT-3)-induced Erk activation and gene transcription (6). Thus, DOC-2/DAB2 acts as a negative feedback regulator in the peptide growth factor-mediated Ras-mitogen-activated protein kinase signal pathway.

In this study, we further dissected the role of other proline-rich domains in the activity of DOC-2/DAB2. We found that the SH3 domain in c-Src, a non-receptor tyrosine kinase, could interact with the first proline-rich domain (amino acid 619–627) of DOC-2/DAB2, and the amount of DOC-2/DAB2/c-Src complex was accumulated in PCa cells shortly treated with EGF. Our results demonstrated that such interaction could lead to the inhibition of tyrosine 416 phosphorylation of c-Src, a key amino acid modulating its kinase activity, in PCa treated with EGF. This interaction further resulted in the downstream effector-Erk inactivation. Apparently, this is a new regulatory mechanism of Src activity mediated by a potent negative factor, DOC-2/DAB2.

EXPERIMENTAL PROCEDURES

Cell Lines, Synthetic Peptides, and Plasmid Constructs—LNCaP, NbE, and COS cells were maintained in T medium supplemented with 5% fetal bovine serum (3). The following peptides were synthesized according to amino acid sequence of DOC-2/DAB2: PPQ (amino acid 619–627); LLQ (amino acid 619–627, proline to leucine); PPL (amino acid 663–671); LLL (amino acid 663–671, proline to leucine); and PPK (amino acid 714–722) (6). All DOC-2/DAB2 cDNA constructs, pCI-neo-T7-p82 (p82) and pCI-neo-T7-ΔN (ΔN), and GST-Grb2 have been described previously (6).

Cell Transfection—For transient transfection, cells were plated 24 h prior to transfection using LipofectAMINE PLUS reagent (Invitrogen). In each experiment, the control plasmid (pCI-neo) was supplemented to make an equal amount of total DNA. Twenty-four h after transfection, cells were switched to serum-free T medium for another 24 h prior to the treatment with 50 ng/ml EGF (Upstate Biotechnology).

For peptide transfection, cells were plated in a 24-well plate with serum-free medium for 24 h. Chariot™ reagent (Active Motif) was mixed with 100 ng of different oligopeptides according to the manufacturer's protocol. One h after transfection, cells were treated with EGF (50 ng/ml), and cell lysate was prepared at the indicated time.

GST Pull-down and Co-immunoprecipitation Assay—For GST pull-down assay, cells were exposed to 50 ng/ml of EGF, and cell lysate was collected in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA supplemented with 1% Triton X-100, and a mixture of protease inhibitors) at the indicated time. After a low speed spin, 0.4 ml of supernatant was separately incubated with either 30 μl of GST-glutathione-Sepharose or GST fusion protein-Sepharose overnight at 4 °C. The next day, the pellet was washed twice with lysis buffer and dissolved in the sample buffer and then subjected to Western blot analysis probed with antibodies against DOC-2/DAB2 (αp96) (Transduction Laboratories) or against T7 tag (αT7) (Novagen).

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† To whom correspondence should be addressed: University of Texas Southwestern Medical Center, Department of Urology, 5323 Harry Hines Blvd., Dallas, TX 75390-9110. Tel.: 214-648-3988; Fax: 214-648-8786; E-mail: JT.Hsieh@UTSouthwestern.edu.

¹ The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PCa, prostate cancer; EGF, epidermal growth factor; SH, Src homology; GST, glutathione *S*-transferase.

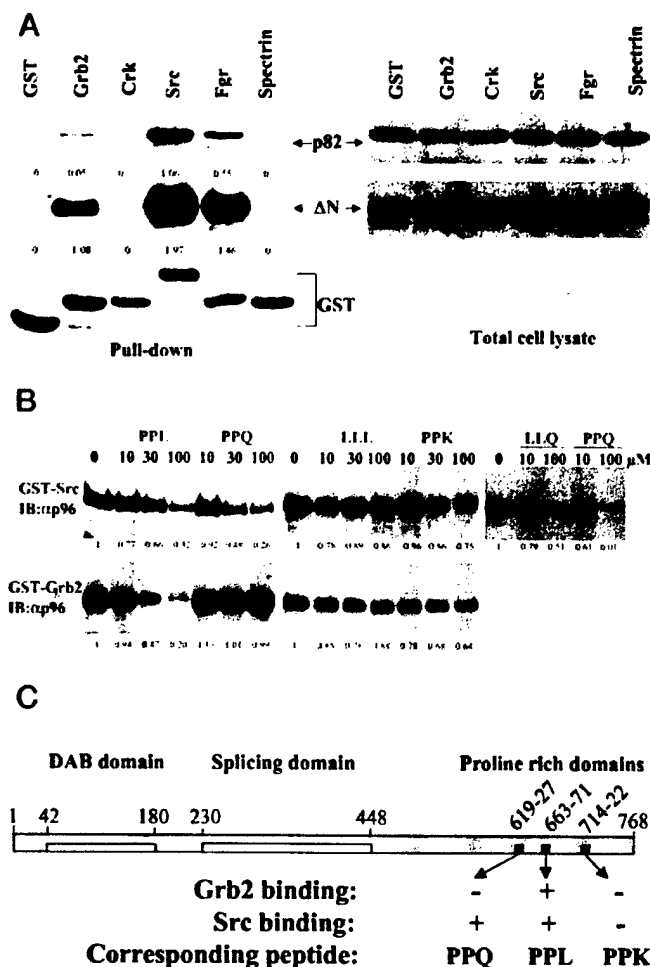


FIG. 1. Differential interaction between the proline-rich domain of DOC-2/DAB2 and several proteins containing SH3 domain. **A**, COS cells ($4 \times 10^5/60$ -mm dish) were transfected with either p82 (top panel) or ΔN (bottom panel) for 48 h. An equal amount of cell lysate was incubated with the indicated GST/SH3 domain fusion proteins. A pull-down assay was performed, and the Western blot was analyzed with αT7. **B**, cell lysate prepared from NbE cells was subjected to a pull-down assay using GST-SH3 (Src) (top panel) or GST-SH3 (Grb2) (bottom panel) with increasing amounts of peptides. The amount of DOC-2/DAB2 from each lane was determined by Western analysis using αp96. The number underneath each lane represented the relative level of DOC-2/DAB2 in each treatment compared with that in control (= 1). **C**, a schematic representation of the SH3 domain in DOC-2/DAB2 interacting with either Grb2 or c-Src.

For co-immunoprecipitation, cell lysate was collected in 0.5 ml of lysis buffer. After a low speed spin, 0.4 ml of supernatant was incubated with 1 μg of antibody against Src (Oncogene Research Products) and then 40 μl of protein A-Sepharose (Amersham Biosciences) overnight at 4 °C. The pellet was washed twice with lysis buffer and dissolved in sample buffer and then subjected to Western blot analysis detected by αT7.

Detection of c-Src and Erk2 Protein Phosphorylation—For determining phosphorylation status of Src, cells were transfected with Src expression vectors and exposed to 50 ng/ml EGF for 10 min. Cell lysate was collected in 70 μl of phosphate-buffered saline (with 1% Triton X-100 and a mixture of protease inhibitors). After a low speed spin, 20 μl of supernatant was subjected to Western blot analysis. The filter was probed with the antibody against phosphorylated Src antibodies against either tyrosine 416 (αpSrc⁴¹⁶) (Upstate Biotechnology) or tyrosine 527 (αpSrc⁵²⁷) (Cell Signaling), and the same filter was stripped and reprobed with the antibody against total Src (αSrc) (Oncogene).

For determining phosphorylation status of Erk2 protein, cells were transfected with hemagglutinin-Erk2 and exposed to 50 ng/ml EGF for 10 min. Cell lysate was collected in 0.5 ml of lysis buffer. After a low speed spin, 0.4 ml of supernatant was immunoprecipitated with hemagglutinin-Matrix (Covance). After washing twice with lysis buffer, the pellets were added with sample buffer and subjected to Western blot analysis. The filter was probed with the antibody against phosphoryl-

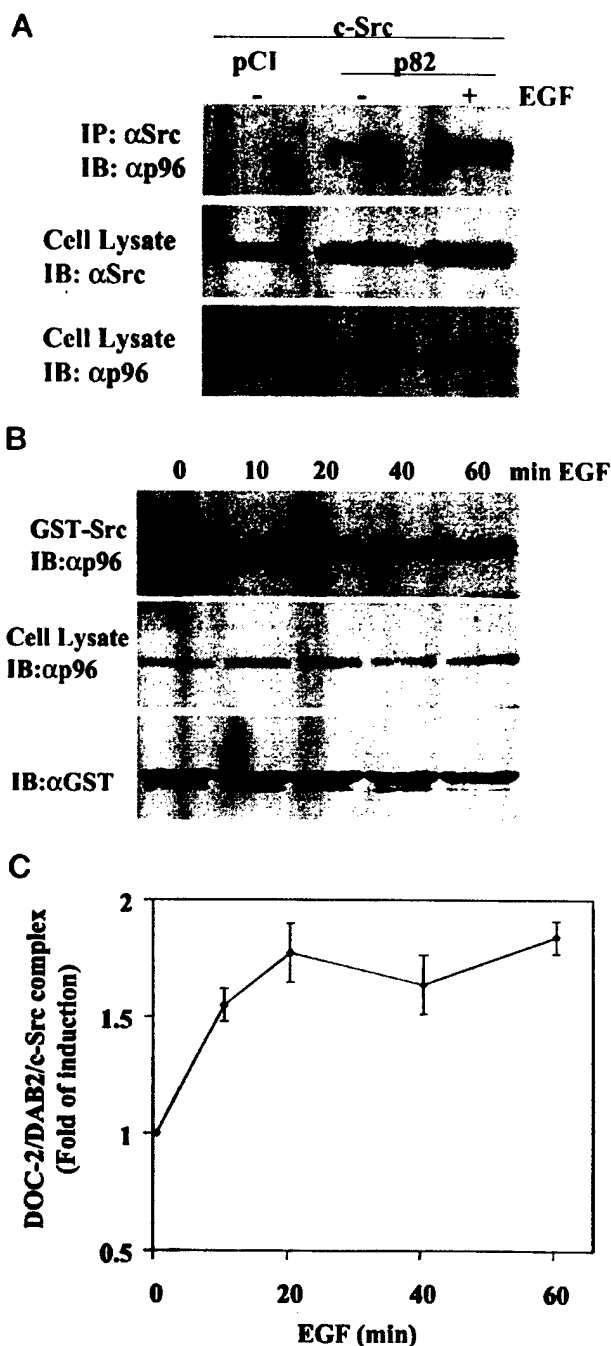


FIG. 2. Increased association of DOC-2/DAB2 with c-Src in cells treated with EGF. **A**, COS cells ($6 \times 10^5/100$ -mm dish) were co-transfected with c-Src and p82 vectors. Twenty min after EGF treatment, cells lysate was subjected to a co-immunoprecipitation assay. **B**, LNCaP cells ($8 \times 10^5/100$ -mm dish) were treated with EGF, and cell lysate was collected at the indicated time. The pull-down was performed using GST-Src and then Western blot was performed using αp96 (top panel). Equal amounts of cell lysate from each sample were analyzed by Western blot using αp96 (bottom panel). **C**, the profile of the DOC-2/DAB2/c-Src complex in LNCaP cells after EGF treatment. Data (mean ± S.E.) were calculated from experiments performed in duplicate.

ated extracellular signal-regulated kinase p44/42 (αErk) (Cell Signaling), and the same filter was stripped and reprobed with antibody against either total extracellular signal-regulated kinase 1/2 (αErk) or p42 (αErk2) (Cell Signaling).

RESULTS

DOC-2/DAB2 Interacts with Src through SH3/Proline-rich Domain Interaction—DOC-2/DAB2 contains three proline-rich

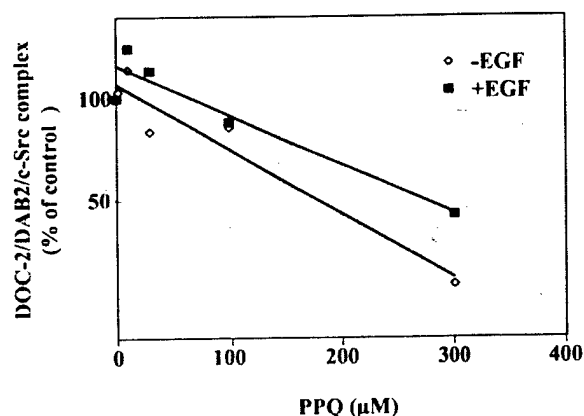


FIG. 3. The effect of EGF effect on the interaction of c-Src and DOC-2/DAB2. COS cells (6×10^5 /100-mm dish) were co-transfected with both c-Src and DOC-2/DAB2 vectors. Cells were collected 20 min after EGF treatment. The co-immunoprecipitation was performed using α Src in the presence of increasing amounts of PPQ and then detected with α 96. The amount of DOC-2/DAB2 was quantified by densitometry, and each data point was calculated based on the ratio between the amount of DOC-2/DAB2 with and without PPQ treatment (= 100%). The IC_{50} was determined as the amount of PPQ needed for 50% of inhibition.

domains indicating that the potential interaction with other proteins containing SH3 domain, in addition to Grb2 (4–6), should be expected. To screen the interaction with proteins containing SH3 domain, we performed pull-down experiment using several GST fusion proteins derived from three groups of proteins containing SH3 domain with different functions. For adapter proteins, Grb2 has been shown to interact with p82 (non-spliced DOC-2/DAB2 protein) previously (4–6); Crk did not show any detectable interaction with p82 (Fig. 1A). For the non-receptor Src tyrosine kinase family, both c-Src and Fgr could interact with p82 (Fig. 1A). However, no detectable interaction was shown between p82 and the SH3 domain of spectrin, an actin-binding protein (Fig. 1A). Apparently, this interaction required the C-terminal of DOC-2/DAB2, because the N-terminal deletion of DOC-2/DAB2 (Δ N) (5) had much stronger interaction compared with p82 (Fig. 1A, lower panel).

We further mapped the specific site of proline-rich domain in DOC-2/DAB2 associated with the Src protein; several proline-rich peptides were synthesized according to the three proline-rich domains in DOC-2/DAB2 (6). As shown in Fig. 1B, peptide PPL corresponding to the second proline-rich domain of DOC-2/DAB2 could interrupt the association of Grb2 with DOC-2/DAB2 in a dose-dependent manner, whereas the control peptide LLL, substitution of all the proline in PPL with leucine did not have any effect (Fig. 1B, bottom panel). Also, the first and third proline-rich peptides (PPQ and PPK) did not have any effect on interaction of Grb2 with DOC-2/DAB2.

On the other hand, both PPQ and PPL, but not control peptide LLL and LLQ, could interrupt the interaction between c-Src and DOC-2/DAB2 (Fig. 1B, top panel). Also, PPK did not have any significant inhibitory effect. These data indicated that the first and second proline-rich domains of DOC-2/DAB2 could interact with c-Src with a similar affinity (Fig. 1C).

EGF Enhances the Association of DOC-2/DAB2 with c-Src—To further verify the interaction of Src with DOC-2/DAB2 intracellularly, we performed co-immunoprecipitation assays. As shown in Fig. 2A, when both c-Src and DOC-2/DAB2 were co-expressed in COS cells, the DOC-2/DAB2 was detected in the immunocomplex precipitated with α Src. The amount of DOC-2/DAB2 in the immunocomplex was substantially increased when the cells were treated with EGF. Similarly, in LNCaP cells, the association of c-Src with DOC-2/DAB2 is

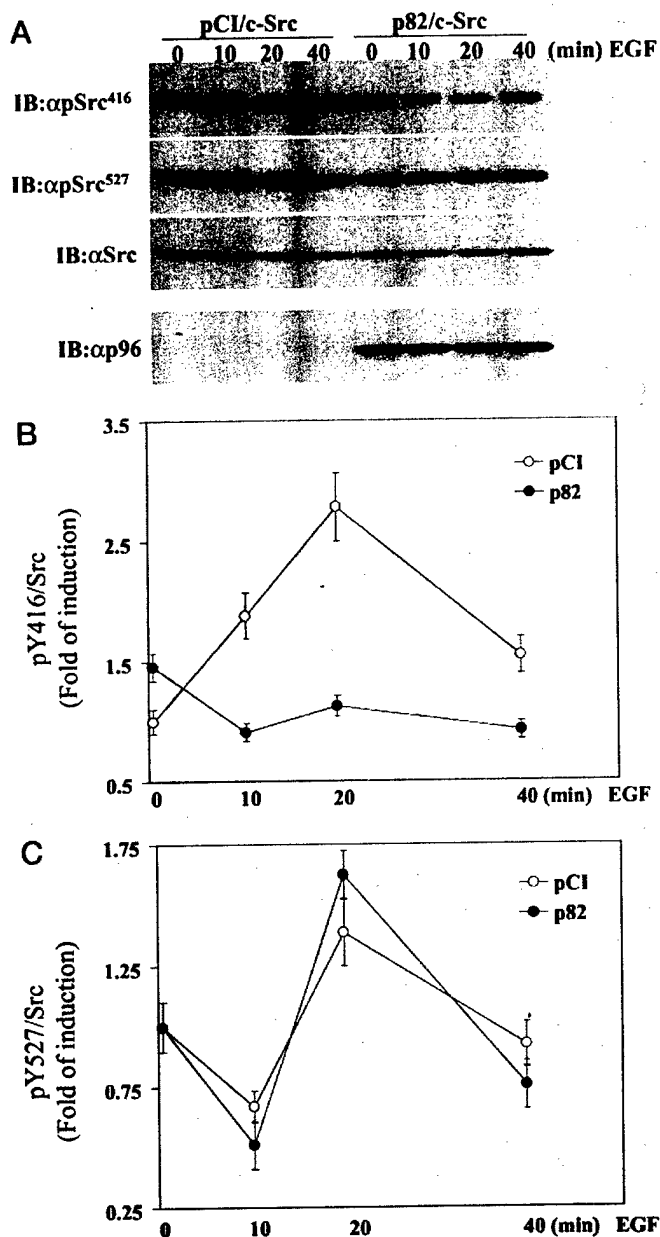


FIG. 4. The effect of DOC-2/DAB2 on the tyrosine phosphorylation of c-Src proteins. A, LNCaP cells (4×10^5 /60-mm dish) were co-transfected with c-Src and p82 vectors. Shortly after EGF treatment, cell lysate was collected at the indicated time. Western blot analyses were carried out using a variety of antibodies. B, the intensity of phosphorylation of Tyr-416 was quantified and normalized with c-Src. C, the intensity of phosphorylation of Tyr-527 was quantified and normalized with c-Src. Data (mean $\pm$ S.D.) were calculated from experiments performed in triplicate.

promptly elevated in a time-dependent manner after EGF treatment (Fig. 2B). The interaction peaked at 20 min after EGF treatment and remained unchanged for 60 min, indicating that c-Src had a prolonged interaction with DOC-2/DAB2.

We further determined whether EGF was able to increase the affinity between c-Src and DOC-2/DAB2. We measured the dissociation constant between c-Src and DOC-2/DAB2 in the presence of PPQ. As shown in Fig. 3, the increasing amount of PPQ caused the dissociation between c-Src and DOC-2/DAB2. However, in the presence of EGF, the IC_{50} of PPQ was 259 μ M compared with the IC_{50} of PPQ (168 μ M) in the absence of EGF. Taken together, these data indicated that EGF could facilitate the interaction between c-Src and

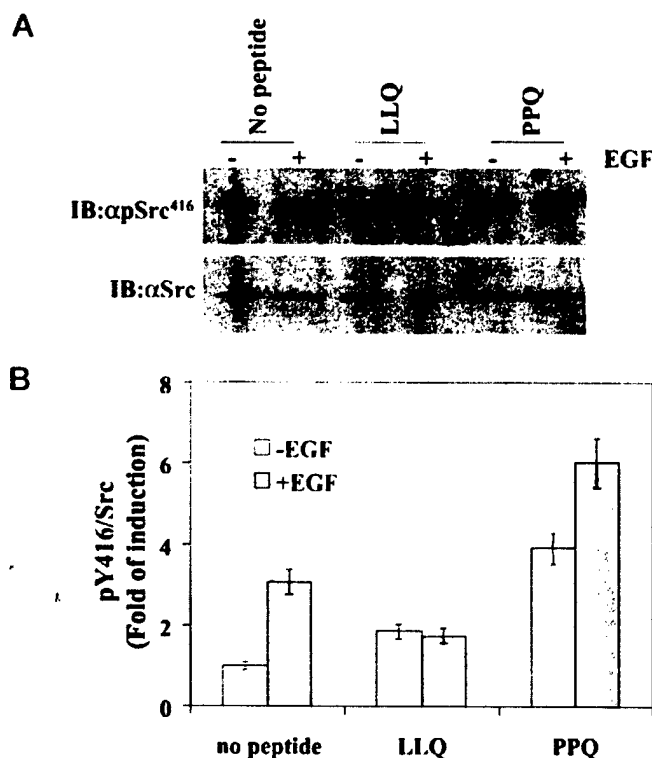


FIG. 5. The effect of proline-rich peptide on the phosphorylation of Src. A, NbE cells (2×10^4 /24-well plate) were transfected with each peptide at 100 ng/well. Twenty min after EGF treatment, cell lysate from each sample was subjected to Western blot analyses using both α pSrc⁴¹⁶ and α Src. B, the amount of phosphorylated Src and the total Src was measured by densitometry. The -fold induction was calculated based on the ratio between phosphoprotein and total protein. Data (mean $\pm$ S.D.) were calculated from experiments performed in triplicate.

DOC-2/DAB2 because of the increased the affinity between both proteins.

DOC-2/DAB2 Inhibits the Tyrosine Phosphorylation of c-Src Protein—It is known that c-Src has two major tyrosine phosphorylation sites. The phosphorylation of tyrosine 416 (Tyr-416) in c-Src represents its activated status; in contrast, the phosphorylation of tyrosine 527 (Tyr-527) in c-Src represents the inactivate form of this protein (11–14). To further determine the effect of DOC-2/DAB2 on the phosphorylation status of c-Src, we examined the phosphorylation status, particularly Tyr-416 and Tyr-527, of c-Src in LNCaP cells. As shown in Fig. 4A, the phosphorylation status of c-Src was determined in LNCaP cells treated with EGF probed with either α pSrc⁴¹⁶ or α pSrc⁵²⁷, respectively, and the total protein levels of c-Src and DOC-2/DAB2 were also determined. In the presence of EGF (Fig. 4B), the phosphorylation of Tyr-416 gradually increased and reached the plateau after 20 min. Concurrently, the phosphorylation of Tyr-527 decreased after 10 min and then rebounded after 20 min (Fig. 4C). Forty min after EGF treatment, both Tyr-416 and Tyr-527 phosphorylation returned to the basal levels (Fig. 4, B and C). In contrast, the presence of DOC-2/DAB2 inhibited the elevated phosphorylation of Tyr-416 after EGF treatment (Fig. 4B). On the other hand, DOC-2/DAB2 did not have any effect on Tyr-527 phosphorylation (Fig. 4C).

To avoid any transfection artifact, we decided to take another approach by interrupting the interaction between DOC-2/DAB2 and c-Src in NbE cells expressing endogenous DOC-2/DAB2 and c-Src proteins using synthetic peptides. PPQ that inhibits the interaction between DOC-2/DAB2 and c-Src (Fig. 2B) was transfected into NbE cells. LLQ with the substitution

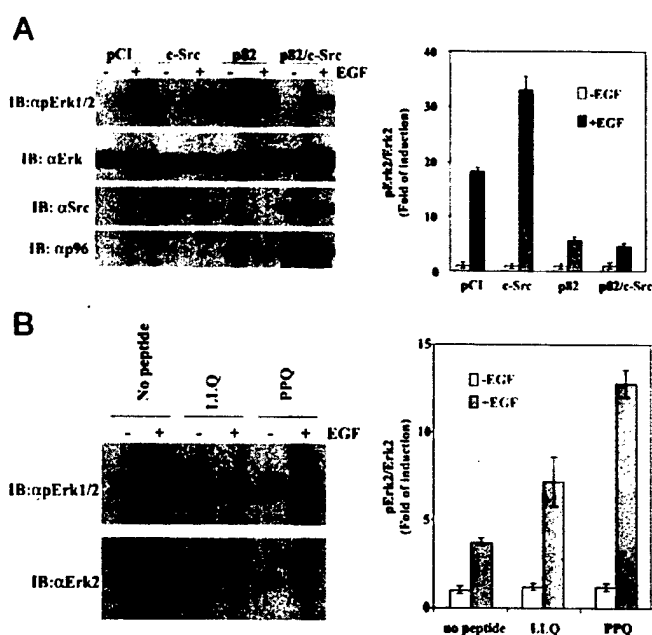


FIG. 6. The inhibitory effect of DOC-2/DAB2 on the c-Src-mediated Erk activation. A, LNCaP cells (4×10^5 /60-mm dish) were co-transfected with plasmid(s) as indicated. Twenty min after EGF treatment, cell lysate from each sample was subjected to Western blot analyses using a variety of antibodies. B, NbE cells (2×10^4 /24-well plate) were transfected with each peptide at 100 ng/well. Twenty min after EGF treatment, cell lysate from each sample was subjected to Western blot analyses using both α pErk1/2 and α Erk2. The amount of each protein was measured by densitometry. The -fold induction was calculated based on the ratio between phosphoprotein and total protein. Data (mean $\pm$ S.D.) were calculated from experiments performed in triplicate.

of proline with leucine was used as a negative control. As shown in Fig. 5A, the phosphorylation of c-Src was determined by using α pSrc⁴¹⁶ in NbE cells after EGF treatment. The intensity of Tyr-416 phosphorylation was measured and normalized with the total Src (Fig. 5B). In the absence of blocking peptide, EGF induced Tyr-416 phosphorylation (~3-fold). Apparently, PPQ could antagonize the interaction between DOC-2/DAB2 and c-Src and significantly increased the Tyr-416 phosphorylation (~4- to 6-fold) in NbE cells without or with EGF treatment. Although LLQ slightly increased Tyr-416 phosphorylation (~2-fold), maybe because of its partial inhibitory activity (Fig. 1B), however, no further elevation in Tyr-416 phosphorylation (~2-fold) was detected in NbE cells after EGF treatment. Taken together, these data indicated that DOC-2/DAB2 could directly suppress the Tyr-416 phosphorylation, but not Tyr-527 phosphorylation, of c-Src via binding to the SH3 domain of c-Src.

DOC-2/DAB2 Inhibits the c-Src-mediated Erk Activation—To understand the effect of the DOC-2/DAB2 on c-Src-mediated signal transduction induced by EGF, the activation of one of the downstream effectors, Erk2, was examined. As shown in Fig. 6A, EGF could stimulate Erk2 phosphorylation in LNCaP cells compared with control. In the presence of c-Src protein, the levels of Erk2 phosphorylation further elevated in LNCaP cells treated with EGF, indicating c-Src elicited an alternative pathway to activate Erk2. Expression of DOC-2/DAB2 suppressed EGF alone induced Erk2 phosphorylation and c-Src-elicited pathway leading to Erk2 phosphorylation. These data indicated that DOC-2/DAB2 was able to inhibit c-Src activity.

The effect of DOC-2/DAB2 on the phosphorylation status of Erk induced by c-Src was also studied in NbE cells. As shown in Fig. 6B, EGF induced an approximately 4-fold elevation of Erk phosphorylation in NbE cells in the absence of peptide.

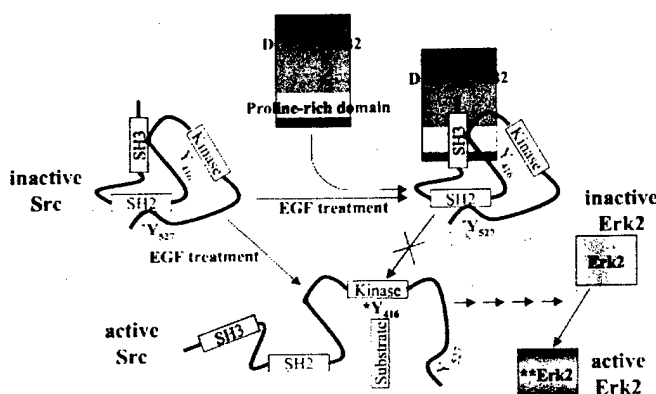


FIG. 7. The role of DOC-2/DAB2 in modulating Src activity. The inactive Src is illustrated by the presence of non-phosphorylated Tyr-416, phosphorylated Tyr-527, and two intramolecular interactions critical for maintaining the closed conformation. The SH3 domain binds to the kinase linker connecting the SH2 domain to the kinase domain. The SH2 domain binds to phosphorylated Tyr-527 (C terminus). The active Src is illustrated by the presence of phosphorylated Tyr-416, non-phosphorylated Tyr-527, and both relaxed intramolecular interactions. The proline-rich domain of DOC-2/DAB2 can interlock the SH3 domain of Src that leads to the suppression of Src kinase activity. Y, non-phosphorylated tyrosine; *Y, phosphorylated tyrosine.

However, PPQ could further enhance the EGF-mediated Erk2 phosphorylation (13-fold). Using the control peptide LLQ, we observed a moderate induction of EGF-mediated Erk2 phosphorylation, because LLQ was a partial antagonist (Fig. 1B). These data provide further evidence that DOC-2/DAB2 is involved in the negative feedback mechanism modulating c-Src activity via the interaction between proline-rich domain and SH3 domain in prostatic epithelia.

DISCUSSION

From our previous studies (5–7), apparently, DOC-2/DAB2 protein can suppress the signal cascade elicited by mitogens via multiple pathways. To delineate the mechanism of action of DOC-2/DAB2 in more detail, we analyzed its interactive protein in this study. From a pull-down assay (Fig. 1A), in addition to Grb2, we further found that Src family proteins were able to interact with the C terminus, but not the N terminus, of DOC-2/DAB2. However, it is interesting to notice that the N terminus of DOC-2/DAB2 contains a disabled motif as in DAB1 phosphorylated by c-Src on its tyrosine residue (15, 16). The phosphorylated DAB1 protein serves as a binding site for SH2 domain of c-Src (15, 16). There is no detectable tyrosine phosphorylation in DOC-2/DAB2 by c-Src (data not shown).² These data suggest a functional difference between DAB1 and DOC-2/DAB2.

Src is a member of a family of non-receptor tyrosine kinases involved in protein receptor tyrosine kinase-mediated pathways (17). In human colon cancer, there is a strong correlation between c-Src protein kinase activity and tumor stage (18). In PCa, c-Src signal transduction is involved in increased migration capacity of these cells (19). We also noticed that almost all PCa cell lines tested in our laboratory expressed c-Src protein, some with absent DOC-2/DAB2 expression (data not shown). These data prompted us to investigate the role of interaction between c-Src and DOC-2/DAB2 in PCa cells under the stimulation of EGF. Our data demonstrated (Fig. 6A) that increased expression of c-Src in LNCaP cells enhanced Erk2 phosphorylation, indicating that the involvement of c-Src in the mitogen-activated protein kinase pathway in PCa cells. In the presence of DOC-2/DAB2, EGF-induced Erk2 phosphorylation was sup-

pressed (Fig. 6A). This could be, at least partially, attributed to the interaction between DOC-2/DAB2 and Grb2 (6). However, DOC-2/DAB2 could also inhibit c-Src-mediated Erk2 phosphorylation (Fig. 6A), implying that DOC-2/DAB2 had an effect on c-Src activity. Data from co-immunoprecipitation assay (Fig. 2A) clearly indicated the DOC-2/DAB2 complex contained c-Src and the major interactive site located in the first proline-rich domain in DOC-2/DAB2. Using a specific peptide, PPQ, the Erk activation can be further enhanced in a prostatic epithelium, expressing endogenous DOC-2/DAB2, treated with EGF (Fig. 6B). These data support the multiple inhibitory roles of DOC-2/DAB2 in EGF-mediated signal cascade.

It is known that there are two major tyrosine phosphorylation sites (Tyr-416 and Tyr-527) in c-Src protein (Fig. 7). However, tyrosine phosphorylation in c-Src has a different impact on its kinase activity (11–14). Tyr-416 phosphorylation, located in the activation loop of Src, is closely correlated with c-Src kinase activity (13, 14, 20). In contrast, Tyr-527 phosphorylation, located in the C terminus of c-Src, associates with the inactive form of c-Src (11–14). X-ray crystallography analyses demonstrate (13, 14, 20–25) that the intramolecular interactions, the binding between SH2 domain and the phosphorylated Tyr-527 and the binding SH3 domain between the kinase linker, are critical for maintaining the inactive status of c-Src. Presumably, changing from a “closed conformation” (inactive) to an “open conformation” (active) signifies c-Src activation. Therefore, deletion or mutation of Tyr-527, often seen in v-Src, leads to constitutive activation of Src kinase activity. Disruption of either intramolecular interaction will lead to activation of c-Src. For example, displacement of SH3 domain with other SH3 binding protein leads to a more profound activation of c-Src activity (26, 27). Also, mutations in SH3 domain have been found in some v-Src proteins (28–30), indicating the key role of SH3 domain in modulating c-Src activity. However, in this study, we demonstrated that DOC-2/DAB2 could interact with the SH3 domain of c-Src and perhaps stabilize its closed conformation. This appears to be a novel mechanism in the modulation of c-Src activity. Because DOC-2/DAB2 acts a potent negative regulator for cell growth, the association of DOC-2/DAB2 with c-Src provides an additional mechanism for its action.

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Signal transduction targets in androgen-independent prostate cancer

Jian Zhou, Jessica Scholes and Jer-Tsong Hsieh

Department of Urology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA

Key words: prostate cancer, androgen-independent prostate cancer, signal transduction, growth factor, tumor suppressor

Abstract

Prostate cancer (PCa) first manifests as an androgen-dependent disease. Thus, androgen-deprivation therapy is a standard regimen for patients with metastatic PCa. Despite the initial success of androgen-deprivation therapy, PCa inevitably progresses from being androgen dependent (AD) to androgen independent (AI), and this marks the poor prognosis of this disease. Relapse of AIPCa becomes life threatening and accounts for the majority of mortality of PCa patients. Currently, no effective therapy is available for controlling AIPCa. Therefore, the challenge in providing a new intervention is to understand the fundamental changes that occur in AIPCa. Increasing evidence indicates that, under androgen-deprived milieu, several signal networks elicited by peptide growth factors dictate the AI phenotype of PCa. This review covers the latest studies investigating the potential involvement of autocrine growth factors in cell proliferation, survival, metastasis, and the reciprocal interaction with the androgen receptor pathway. In addition, loss of the negative feedback mechanism of the signal cascade further amplifies the effect of growth factors, and thus contributes significantly to the onset of AIPCa. The understanding of the signal target(s) in AIPCa should provide the new markers for prognosis and a new strategy for prevention and therapy.

Progression of androgen-independent prostate cancer

Clinical observations [1–3] indicate that eunuchs and prepubertal castrates do not develop prostate cancer (PCa). This suggests that all the steps of PCa carcinogenesis are prevented by prostatic atrophy associated with early castration or androgen deprivation. Animal models, first developed by Noble [4] and Pollard and Luckert [5] in which chronic administration of androgen and/or estrogen to certain strains of intact male rats caused PCa, further support these observations. Current effective therapeutic modalities, first developed by Huggins and Hodges in 1941 [6], interrupt the positive effect of growth stimulation by androgen. Androgen thus appears to be a 'pure' mitogen for the growth of PCa cells. Conversely, the morphogenic effect of androgen on normal prostatic epithelium must be impaired during the malignant process.

Despite the initial responsiveness of PCa toward androgen ablation, tumor cells invariably relapse to

an androgen-refractory state that ultimately leads to mortality. Studies from the Shionogi mouse model [7] support the observation that androgen deprivation leads to a 90% regression of tumor mass (mainly androgen-dependent cells). But, recurrent tumors have a 500-fold increase in the number of androgen-independent (AI) cells over the fraction measured in the parent tumor. Using proliferation-associated antigens (Ki-67, PCNA, MIB 1) as markers, Bonkhoff and Remberger [8] estimated that approximately 70% of the proliferative activity is confined to basal cells in both normal and hyperplastic prostatic epithelia.

AIPCa cells thus appear resistant to a majority of chemotherapeutic agents that target rapidly cycling cells. These data indicate that the androgen eliciting differentiating pathway is often impaired in AIPCa, which may derive from the malignant transformation of 'stem cells' in the normal gland. Based on these findings, we believe that an effective therapy for AIPCa should focus on restoring the differentiation pathway that is operative in normal prostatic epithelia, but is often impaired in AIPCa cells.

Although the stem cells in the prostate are not well characterized, it is believed that certain basal cells may possess stem cell properties. Shortly after androgen deprivation, luminal epithelial cells in the prostate undergo apoptosis; the remaining epithelial cells are the AI basal cell population. Androgen administration can restore the normal acini/ductal structure and function in an involuted prostate by promoting the growth and differentiation of the remaining basal cell population [8,9]. Even after repeated administration of androgen to castrated animals, the prostate always re-grows to a previously programed organ size. This suggests that a limited number of stem cells from the basal cell population determine the ultimate growth potential of the gland [8-11].

The molecular signal(s) involved in this process are likely important in maintaining the homeostasis of the prostate gland. Imbalance in the homeostatic control of the signaling cascade in the stem cell may underlie the malignant phenotype of AIPCa cells. To better understand the biologic properties of AIPCa cells, we will elucidate: (a) the role of several key peptide growth factors that can stimulate cell growth, survival, and metastasis, (b) the intracellular pathways responsible for these processes, and (c) the cross-talk between these pathways and the steroid hormone-elicited pathway. In addition, we will discuss the potential impact of the loss of the negative feedback mechanism associated with these pathways on recurrent AIPCa.

Mitogenic signal pathways in AIPCa

Altered production of growth factors and/or aberrant expression of their receptors are usually associated with PCa cells. Increasing the production of autocrine growth factors is an important step for the appearance of AIPCa after androgen deprivation. For example, epidermal growth factor (EGF) is a mitogen required for normal prostate epithelial cells in both human and rat [12,13], and it is present, in a large amount, in human prostatic fluid [14]. Blocking EGF receptor (EGFR)-elicited signaling can inhibit the proliferation of both DU145 and LNCaP cells, which indicates the important involvement of the EGFR signal axis in the growth of PCa [15].

In the normal gland, the transforming growth factor- α (TGF- α), a ligand for EGFR, predominantly expresses in stromal cells [16]. However, the EGFR expresses in human prostatic epithelial cells with a higher expression in basal cells than luminal

epithelia [17]. This suggests that TGF- α and EGFR have a paracrine interaction. In contrast, the autocrine interaction of EGF/TGF- α and its receptor has been shown to play an important role in the progression of PCa [18-20]. Particularly, increased autocrine production of EGF and/or TGF- α was found in several AIPCa cell lines including DU145. This caused the activation of EGFR as demonstrated by high levels of autophosphorylation of EGFR [19,21]. Furthermore, the addition of anti-EGFR antibody to DU145 cells can reduce EGFR autophosphorylation and subsequently inhibit cell proliferation [22].

In other cases, changing the receptor affinity in PCa allows cancer cells to utilize their own autocrine growth factor. In the Dunning tumor model, AT3 tumor cells expressed a different subclass of fibroblast growth factor receptor (FGFR) protein by switching exon IIIb (high affinity to keratinocyte growth factor [KGF]) to exon IIIc (high affinity to acidic FGF [aFGF] and basic FGF [bFGF]) as they acquire a more aggressive phenotype [23]. Similar exon switching has also been found in DU145 and its xenograft [24]. It is also found in PCa specimens, although incidence is low [25].

Autocrine production of FGF has been associated with the proliferation of AIPCa [23,26,27]. Moreover, AI tumors from Shionogi mice produce a bFGF-like protein [28]. In the Dunning tumor model, in concert with the switching of the receptor subtype in AT3 tumors, the increased steady-state levels of FGF-2, FGF-3, and FGF-5 mRNA were also found in these tumor cells [23].

Nerve growth factor (NGF) also appears to be a mitogen for AIPCa. For example, human prostate cell lines (TSU-Pr1, DU145, PC3, and LNCaP) are sensitive to NGF for proliferation [29,30]. Nevertheless, LNCaP does not produce NGF [31]. Since normal prostate stromal cells produce several active forms of NGF [32,33], this suggests that paracrine NGF could be a potent factor necessary for the growth of primary PCa. However, other AIPCa cell lines such as DU145, PC3, and TSU-Pr1 produce NGF in an autocrine manner [31], which indicates that paracrine and/or autocrine production of NGF contributes to the growth of AIPCa.

Immediately after autocrine growth factors bind to their specific receptor (protein receptor tyrosine kinase, PRTK), dimerization and autophosphorylation of the receptor promote interactions with cytoplasmic proteins. These interactions initiate a cascade of phosphorylation events through a variety of adapter proteins and kinases, which transduce the mitogenic signal by increasing gene expression in the nucleus (Figure 1).

Ras (V12*ras*) enhances ERK activation, cyclin D1 induction, and proliferation in LNCaP cells [45,46]. Moreover, suppressing RAS function by inhibiting its protein farnesylation using a peptidomimetic inhibitor (L-744,832) leads to a significant delay in the development of PCa in a xenograft model [47]. These data indicate that RAS protein plays a critical role in the progression of PCa. Nevertheless, *Ras* gene mutation is rare in PCa [42–44,48–50]. This implies that some other factor(s) may be involved in increasing RAS protein levels in PCa.

It also appears that RAF plays a functional role in PCa cells. Suppression of *Raf* gene expression by an anti-sense oligonucleotide induces apoptosis in PC3 and reduces tumor formation in nude mice [51,52]. Surprisingly, prolonged activation of RAF and MAPK are also able to induce cell cycle arrest in LNCaP cells through induction of p21^{WAF1/CIP1} [53] or apoptosis [54]. These data suggest that perturbing a delicate balance of each component of the mitogenic signal cascade could adversely effect cell growth.

Overwhelming data demonstrate the critical role of the MAPK pathway in cell growth and malignant transformation [55–60]. In several PCa cell lines such as LNCaP and DU145 cells, inactivation of MAPK (e.g. ERK) by interrupting EGF binding to EGFR using a flavonoid antioxidant (Silibinin), decreases DNA synthesis and cell growth [61]. Using anti-phosphorylated ERK antibody, heightened activation of MAPK is often detected in high-grade PCa and AIPCa [59]. In AIPCa cell line such as DU145 cells, the constitutive phosphorylation of ERK2, a hallmark of MAPK activation is also observed [15]. However, this ERK2 activation can be blocked by several EGFR inhibitors such as Tyrphostin AG1748 and Mab-EGFR-528, indicating that the EGFR-elicited signal axis is critical for activating the MAPK pathway in AIPCa [15]. Therefore, it is likely that the increased activation of MAPK in high grade PCa and AIPCa is due to the stimulation of autocrine growth factors, which provides a growth advantage for the progression of these cancer cells.

Activation of cyclin D1, a key downstream effector protein in both MAPK and the protein kinase C (PKC)-elicited signal pathway, prompts cells entering S phase during cell cycle. For example, in LNCaP cells, both EGF and TPA (a PKC activator) can induce cyclin D1 in LNCaP cells [62]. Overexpression of cyclin D1 in LNCaP cells results in accelerated cell growth and increased *in vivo* tumorigenicity [63]. However, unlike breast cancer, cyclin D1 gene amplification is relatively rare in PCa cell lines [64]. Using quantitative

RT-PCR and Western analyses, Gumbiner et al. [65] demonstrate that no apparent increased cyclin D1 transcript and protein levels were observed in four prostate tumor cell lines and their xenograft tumors. However, an increased cyclin D1 transcript level was found in a small subset (4 out of 96) of clinical specimens derived from either stage C and D [65]. In another study using immunohistochemistry, cyclin D1 positive tumor (defined as identification of positive immunoreactivity in the nuclei of >20% of tumor cells) is 11% (10 of 86) of the primary cases compared with 68% for AI bone metastatic lesion prostate [66]. These data indicate that cyclin D1 may be involved in the onset of AIPCa.

Cell survival signals in AIPCa

Most mitogenic signals have a dual function involved not only in cell proliferation but also cell survival. For example, inhibition of MAPK activity by either Tyrphostin AG1748 (EGFR inhibitor) or PD98059 (MAPK inhibitor) enhances the G2/M cell cycle arrest and radiation-induced cell killing [67]. In addition, PTEN and the phosphatidylinositol triphosphate kinase (PI3-K) pathway have been implicated in the regulation of G1 growth arrest [68] and the regulation of cell survival. PTEN, a dual phosphatase for both phosphatidylinositol 3,4,5-triphosphate (PIP3) and tyrosine phosphoprotein, is frequently lost or mutated in AIPCa [69–72]. Loss of PTEN expression, mostly due to down-regulation of the gene by DNA hypermethylation, is found in PCa [72]. In the absence of PTEN, PIP3 phosphorylated by PI3-K accumulates in cells and it is an activator for Akt/PKB kinase, which promotes cell survival [73–75].

Recent data indicate that dephosphorylation of phosphoinositol-triphosphate (PI3P) and focal adhesion kinase (FAK) by PTEN promotes apoptosis through inactivating the PI3-K/Akt cell survival pathway [74,75]. Also, activation of Akt can prevent TRAIL-induced apoptosis in LNCaP cells [76]. On the other hand, in LNCaP cells, the constitutive activation of PI3-K pathway due to PTEN mutation contributes to cell survival since inhibition of this pathway can cause cell apoptosis [77]. However, some data indicate that the PI3-K inhibitor-induced apoptosis in LNCaP cells can be reversed by activating EGFR and/or the androgen receptor (AR) [78], which suggests that the some pathway(s) parallel with the Akt/PKB pathway in PCa also contributes to cell survival.

Signal pathways involved in the metastasis of PCa

PCa cells have a high propensity to metastasize to bone, at which point AIPCa can arise and becomes a life-threatening disease. Metastasis requires not only cell mobility, but also the interaction between tumor cells and their surrounding environment. Several studies demonstrate that a growth factor and its receptor may be involved in this process. For example, overexpression of EGFR can increase the *in vivo* metastasis potential of DU145 cells [79]. Overexpression of Her-2/neu, a member of EGFR family, can facilitate the metastasis of a nontumorigenic rat prostate NbE cells to skeletal muscle in the rib [80]. Conversely, using EGFR-specific kinase inhibitor (PD153035) can reduce invasiveness of PCa using a transgenic adenocarcinoma mouse prostate (TRAMP) model [81]. These data indicate that the involvement of EGFR signaling in the metastasis of PCa.

The possible underlying mechanism for growth factor(s)-elicited PCa metastasis is due to the induction of cell migration. Rajan et al. [82] report that an EGF-like protein identified from several bone and leukemia cell lines acts as a potent chemoattractant, which increases cell migration of a PCa cell line (TSU-Pr1). This study suggests that the EGF-like molecule attracts PCa to invade or metastasize the peripheral lymph nodes and medullary bone. Concurrently, activation of the MAPK pathway is often associated with metastatic PCa [83,84]. It has been shown that the MAPK inhibitor can suppress the expression of the $\alpha 6$ integrin gene, a critical receptor for the interaction with matrix protein by the metastatic PCa, in both PC3 and LNCaP cells [85].

In LNCaP cells, increasing survival can also increase metastatic potential [86]. Conversely, PTEN can inhibit the PI-3K/Akt pathway by dephosphorylating PIP3, which leads to reduced cell motility [87,88]. Furthermore, PTEN can also inhibit cell migration by directly dephosphorylating FAK [89,90] and SRC homolog and collagen protein (SHC) [91] (Figure 1). The steady-state levels of FAK in three PCa cells (LNCaP, PC3, DU145) correlate with the cell migration capability [92]. Moreover, presence of the dominant negative FAK and inhibitor of SRC (oncogenic protein of Rous Sarcoma virus) protein can significantly inhibit migration of PCa [92], indicating that the involvement of integrin/matrix via SRC/FAK signaling is a key determinant for PCa metastasis. Similarly, overexpression of dominant negative SHC can inhibit

cell migration in a PTEN-negative glioblastoma cell-U-87MG [93].

Cross-talk between steroid hormone and growth factor-elicited signal pathways

Androgen is known as a key mitogen for primary PCa. In an androgen deprived milieu, it is possible that the AR in AIPCa can function through a ligand-independent fashion. Some data indicate that the ligand independent activation of AR could be achieved by mutation occurring in the ligand binding domain of AR and/or by associating with the growth factor-mediated signaling pathway. In AIPCa, AR mutation and amplification have been found in 20–40% of cases [94–96]. Also, increasing evidence indicate that there is interaction between AR and the peptide growth factor receptor signaling pathway. Activation of MAPK and protein kinase A (PKA) pathways can lead to the phosphorylation of AR, which increases its interaction with cofactors such as ARA50 or ARA70 and consequently enhance the transcription activity of AR [97,98]. This is consistent with data showing that the transcription activity of AR in LNCaP cells can be activated by several growth factors (insulin-like growth factor-1 [IGF-1], KGF, and EGF) [99] capable of initiating the MAPK pathway. Also, overexpression of Her-2/neu can induce AR activation through activation of the MAPK phosphorylation cascade [100,101]. Moreover, Sehgal et al. [102] indicate that androgen can induce amphiregulin, a ligand for EGFR, which could activate EGFR-mediated signal transduction. These data clearly indicate that reciprocal interaction between the AR and MAPK-mediated pathways may underlie the AI growth of PCa.

Loss of homeostatic control of the signal pathway in AIPCa

Despite the prevalence of positive signal(s) involved in the relapse of AIPCa, loss of negative feedback control in the signal network also significantly impact the onset of AIPCa. Loss of the tumor suppressor PTEN (Figure 1) has been implicated in proliferation, cell survival, and metastasis of PCa cells. The dual phosphatase activity of PTEN can dephosphorylate PIP3 which is required for activating the PI3-Kinase/Akt pathway implicated in promoting proliferation and cell survival in PCa [77,78]. PTEN can also suppress cell

proliferation, survival, and migration of prostate cells by dephosphorylating tyrosine phosphoprotein such as FAK and SHC [89–91].

Based on animal models and clinical observation, we believe AIPCa possesses similar stem cell properties as the normal prostate gland. To unveil the fundamental changes that occur in AIPCa, we hypothesized that altered homeostatic control machinery, operative in normal prostatic basal cells, underlie the malignant phenotype of AIPCa. Our laboratory has screened cDNAs from the enriched basal cell population of the degenerated prostate gland. A candidate gene has recently been identified as DOC-2/DAB2 (differential-expressed in ovarian cancer-2/disabled 2), which was cloned from differential display as a potential tumor suppressor in ovarian cancer [103]. It encodes an 82 kDa phosphoprotein with a highly conserved sequence between human and rodent, which implies its important biological function. The N-terminal of DOC-2/DAB2 contains a homology domain (i.e. DAB domain) with the *disabled* (SRC-binding protein) gene involved in the differentiation of the neuron [104]. The C-terminal of DOC-2/DAB2 contains three proline-rich domains that can bind to SH3-containing protein [105]. Therefore, DOC-2/DAB2 appears to be a typical molecule involved in the signal network.

In the normal prostate, DOC-2/DAB2 is predominantly associated with basal epithelia cells when the prostate undergoes androgen-deprived degeneration [103]. Decreased expression of DOC-2/DAB2 has been found in several tumors including ovarian, choriocarcinoma, breast, and prostate. We also observed the absence of DOC-2/DAB2 in several PCa cell lines derived from AIPCa patients [103]. Increased expression of DOC-2/DAB2 can suppress cell growth and increase G1/Go growth arrest in a tumorigenic LNCaP subline (C4-20 cell). Similar results were observed in other cancer types [106–108]. In ovarian cancer, reintroducing DOC-2/DAB2 into cancer cells can reduce cell growth, tumorigenicity and suppress the serum induced *c-fos* gene expression [106–109]. These data indicate that DOC-2/DAB2 is a potent tumor suppressor.

The mechanism responsible for the loss of DOC-2/DAB2 in PCa is not fully understood. However, some evidence indicate that the expression of DOC-2/DAB2 is suppressed by the RAS-mediated pathway because DOC-2/DAB2 is down regulated at least 100-fold in RAS-transformed cells and such suppression can be reverted in the presence of MAPK inhibitor PD98059 [110]. Also, the gene transcription

of DOC-2/DAB2 can be modulated by either GATA-6 or retinoic acid during embryonic differentiation [111,112]. Noticeably, retinoic acid treatment can cause apoptosis in PCa cells [113,114]. However, the relationship of DOC-2/DAB2 and retinoic acid-induced apoptosis in PCa remains undetermined. Moreover, our data also demonstrate that DOC-2/DAB2 is induced during TPA-induced megakaryocyte differentiation of K562 cells [115]. Based on these findings, we believe that the regulation DOC-2/DAB2 gene is associated with cell differentiation in several cell types including prostatic epithelia.

To examine the role of DOC-2/DAB2 in modulating signal transduction which may impact the phenotype of AIPCa cells, we first demonstrated that the presence of DOC-2/DAB2 is able to inhibit TPA-induced gene expression [116]. Moreover, DOC-2/DAB2 (Figure 1) can be rapidly phosphorylated by treatment of growth factor and TPA [116]. Therefore, we examined the impact of DOC-2/DAB2 phosphorylation on the effect of TPA in PCa cell lines such as LNCaP cells. It appears that the serine 24 in the N-terminal of DOC-2/DAB2 is the key phosphoamino acid residue in modulating PKC-elicited signal pathway, since the alteration of this residue abolishes the activity of AP-1 induced by TPA [115].

Recently, we identified a novel RAS-GTPase-activating protein (RAS-GAP) as a DOC-2/DAB2 interactive protein (DIP1/2) [117]. The human DIP1/2 gene locates at 9q33.1–33.3 [118] proximal to a potential tumor suppressor gene, TSC1 (Tuberous Sclerosis) [119]. It has also been reported [120] that a novel RAS-GAP gene fused to a myeloid/lymphoid leukemia gene in acute myeloid leukemia with chromosomal translocation [t(9;11)(q34; q23)]; DIP1/2 may be that candidate gene.

Using detailed biochemical analyses, we demonstrated that DIP1/2 is a typical RAS-GAP that hydrolyzes RAS-GTP (active RAS) to become RAS-GDP (inactive RAS). We further demonstrated that the interaction between DOC-2/DAB2 and DIP1/2 enhance the GAP activity in PCa cells (Figure 1), and, decreased expression of DIP1/2 is associated with several PCa cell lines derived from AIPCa patients. Apparently, one of the mechanisms of action of DOC-2/DAB2/DIP1/2 is to modulate RAS activity in AIPCa. In addition, the DAB domain of DOC-2/DAB2 directly associates with Smad and mad-related protein 2 and 3 (Smad2 and Smad3), which restores the transforming growth factor- β (TGF- β) signaling pathway in TGF- β mutant cells [121]. Thus, DOC-2/DAB2

appears to have multiple mechanisms of action that modulate the growth/differentiation of mammalian cells.

As shown previously, DOC-2/DAB2 interacts with GRB2 in mouse macrophage treated with colony stimulating factor-1 [105]. We further analyzed the functional role of the C-terminal of DOC-2/DAB2, particularly the proline rich domain, in growth factor-elicited signal transduction. In PCa, the expression of DOC-2/DAB2 prevents the SOS from binding GRB2 (Figure 1), which leads to the suppression of MAPK activation initiated by EGF [122]. Similar action of DOC-2/DAB2 is also observed in PC12 cells stimulated with neurotrophin NT3 [122], suggesting that DOC-2/DAB2 is involved in the growth/differentiation of neuronal cells. Moreover, our preliminary data indicated that some other SH3-containing proteins such as SRC and NCK can interact with the DOC-2/DAB2 (Figure 1). The biologic implication of this interaction warrants further investigation. Nevertheless, the DOC-2/DAB2 complex represents a unique negative feedback machinery for balancing the signaling cascade elicited by exogenous stimuli. Altered expression of this complex underlies the onset of AIPCa.

Concluding remarks

Relapse of AIPCa signifies that PCa has become autonomous after androgen deprivation; the presence of peptide growth factors or cytokines becomes a prevalent mitogen for AIPCa. However, it is clear that these exogenous stimuli elicit various signal networks critical for cell proliferation, survival, and metastasis. Among these pathways, increased MAPK activation appears to predominate in the AIPCa cells. This is evidenced by several molecular markers such as RAS, ERK, and cyclin D1. Overwhelming *in vitro* and *in vivo* data demonstrate this relationship. Moreover, recent data also indicate the MAPK and AR pathways have a reciprocal interaction, which underlies the mechanism for the AI progression. Nevertheless, unlike other cancer types, the mutation rate of these key effectors (such as *Ras* and *cyclin D1*) is relatively low in PCa. Therefore, it is likely that other regulatory pathways such as the negative feedback mechanism are impaired in the PCa cells.

Several molecules were identified as a part of a negative regulatory network. PTEN is an excellent example of such a molecule because, (a) decreased or frequent loss of PTEN expression is found in PCa, and (b) loss

of PTEN is known to increase the cell survival of PCa through the PI-3K/Akt pathway or through cell migration/metastasis through the SRC or FAK pathway. In addition, our laboratory identified a unique complex (DOC-2/DAB2/DIP1/2) that modulates the RAS and TGF- β mediated pathway. Since this complex can associate with many other effectors, it is possible to predict the potential impact of this complex on the progression of AIPCa.

The ultimate goal in studying the signal network in AIPCa is to identify marker(s) for early prognosis of AI disease and to develop specific agent(s) for disease intervention. Recent clinical approval of the c-ABL tyrosine kinase inhibitor (STI-571, Gleevec) marks the beginning of target-specific cancer therapy. In addition to the completion of the human genome project, in the foreseeable future, cancer therapy could be customized based upon an individual patient's genetic profile. Therefore, profiling signal transduction targets in AIPCa has tremendous clinical application.

Key unanswered questions

1. It appears that AIPCa contains a heterogeneous cell population. What are the critical signal networks operative in AIPCa cells?
2. AIPCa has a high propensity to grow in bone. What are the critical signal networks responsible for the interaction between these cells?
3. There is a significant overlapping in the signal cascade-elicited by many biologic responses. Are there key merging points in AIPCa?
4. What is the impact of different nerve growth factors on the progression of AIPCa?
5. It appears that a close interaction exists between AR and MAPK pathways. Can DOC-2/DAB2 complex modulate the AR-mediated signal transduction?
6. The presence of negative regulator is to prevent the constitutive activation of positive signal cascade elicited by stimuli. Therefore, what is the mechanism resulting in the association between these proteins immediately after the initial activation?
7. Loss of DOC-2/DAB2 expression is often found in AIPCa. What is the underlying mechanism(s) leading to the loss of DOC-2/DAB2 expression?
8. The underlying mechanism of negative feedback pathways is somewhat similar. Can these negative feedback pathways complement each other in AIPCa?

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Address for offprints: JT Hsieh, Department of Urology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9110, USA; *Tel:* 214-648-3988; *Fax:* 214-648-8786; *e-mail:* jt.hsieh@utsouthwestern.edu