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**13. ABSTRACT** *(Maximum 200 Words)*
The goal of this study to define the signal pathway(s) elicited by two homeostatic genes, C-CAM1 and DOC-2, in the basal cells of normal prostate. Prostate basal cells have been shown to have similar biologic properties to the androgen-independent (AI) prostate cancer. The outcome of this study can improve the current treatment strategy for AI prostate cancer. Structurally, C-CAM1 is a cell adhesion molecule with receptor kinase motif in the C-terminus. Together with DOC-2, a typical signal molecule, we believe that both genes play an important role in controlling the growth and differentiation of prostatic epithelia. Our results indicate that both C-CAM1 and DOC-2 are down-regulated in AI prostate cancer and increased expression of individual gene can inhibit cancer growth. We have further defined the functional domain in both genes which modulate their tumor suppression activities. More detailed analyses are underway for C-CAM1 protein. For DOC-2 protein, it appears that DOC-2 can interact with an novel protein that belongs to the Ras GTPase-activating protein (GAP) family. Ras-GAP is a key factor to maintain the balance of both active and inactive status of Ras protein. Our finding signifies the critical role of DOC-2 in prostate development and carcinogenesis.

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# INTRODUCTION

The purpose of this study is to define the signaling pathways which control growth and differentiation of prostatic epithelia. Homeostatic factor(s) that operate in the androgen-independent (AI) basal cell population of the prostate can modulate the stimuli for extracellular growth by regulating particular gene(s) expressed in the nucleus. Genes with a potent growth inhibitory effect on prostate cancer, C-CAM1 and DOC-2, were chosen for the study because C-CAM1 behaves like a membrane receptor and DOC-2 is a signaling molecule. Moreover, both are novel genes, which have recently been reported by this laboratory. To delineate the signaling network elicited by these genes, three aims were proposed: 1) to specify the interaction of C-CAM1 and DOC-2 during development of the prostate and of carcinogenesis; 2) to identify the signaling pathway elicited by these putative homeostatic molecules; and 3) to document the effect of microenvironmental factors on the regulation of these molecules. Recurrent AI prostate cancer has been shown to possess many similar characteristics with the population of AI basal cells in the normal prostate. Thus, the larger purpose of this study is to apply the knowledge gained to develop an effective regimen to treat AI prostate cancer.

# RECENT PROGRESS

The study had progressed smoothly in the past year. One manuscript has been published (Appendix 1); a second is in preparation. Overall, Task 1 is nearly completed, Task 2 is 50% completed, and work on Task 3 will begin shortly. Detailed progress of these aims is outlined below.

*Task 1.* To specify the interaction of C-CAM1 and DOC-2 during development of the prostate and of carcinogenesis.

Recently, we demonstrated that expression of C-CAM1, an immunoglobulin (Ig)-like CAM, correlates with androgen-induced prostate epithelial differentiation in an organ-specific manner (1). Constitutive expression of the C-CAM protein was detected in normal prostatic epithelium throughout the fetal to adulthood stage (2). However, C-CAM expression was diminished in both prostate intraepithelial neoplasia and cancer lesions (2), which indicates that loss of C-CAM expression may be involved in the early stages of prostate carcinogenesis. In addition, increased C-CAM expression, through gene transfection or delivery of recombinant adenovirus, can effectively control growth of prostate cancer both *in vitro* (3) and *in vivo* (4). According to sequence analysis of C-CAM1 cDNA, C-CAM1 represents a unique CAM with a potential signal transducing capability. Therefore, the relationship of each C-CAM structural domain that could possibly affect its tumor suppression function *in vivo* was analyzed using a variety of mutants – ranging from deletion mutation of the extracellular domain to the intracellular domain. Because of its high infectivity, an adenoviral vector system was employed as a delivery sytem. Data from *in vivo* tumorgenic assay indicated that the C-CAM mutant without cell adhesion function retained its tumor suppressive activity. In contrast, deletion in the Ser/Thr phosphorylation site but not in the tyrosine phosphorylation site of C-CAM1 resulted in the loss of tumor suppressive activity. These data suggest that, in contrast to the extracellular domain, the potential Ser/Thr phosphorylation site in the intracellular domain of the C-CAM molecule and its associated protein(s) are crucial for the suppression of the growth of prostate cancer (see Appendix 1).

Elevated levels of DOC-2 mRNA and protein are detected in the degenerated prostate (5). Immunohistochemical staining indicates that both DOC-2 and C-CAM1 proteins are associated with the enriched basal cells in the prostate (1,5). Furthermore, restored expression of DOC-2 can inhibit the growth of prostate cancer (5). It is likely that both proteins have an interaction. So, to further delineate the possible interaction between DOC-2 and C-CAM1, a co-immunoprecipitation experiment was conducted by transfecting both DOC-2 and C-CAM1 expression vectors. As Fig. 1 demonstrates (Appendix 2), no physical interaction between these proteins can be detected. This suggests that DOC-2 and C-CAM1 proteins may not contact directly.

*Task 2.* To identify the signaling pathway elicited by these putative homeostatic molecules.

Our previous data demonstrated that the presence of an unknown factor(s) was associated with DOC-2 protein by immunoprecipitation. We used a yeast two-hybrid system (6) to search for these factors. Initially, the bait vector was constructed from pVJL11 by inserting the N-terminus of DOC-2 protein (i.e., residues 1-269), which had been demonstrated to the major protein phosphorylation site in DOC-2. Among an estimated $9 \times 10^4$ clones plated for screening, 94 clones with His$^+$ phenotype were obtained. But, only 34 clones appeared positive in both His and LacZ phenotypes. After screening with mating test to rule out false positive results, 3 independent clones, DIP1 (1.7 kb), DIP2 (5.5 kb), and DIP7 (2.8 kb) were confirmed. Further cDNA sequencing and alignment (Appendix 3) suggest these clones belong to, and are novel members of, the GTPase activating protein (i.e., GAP) family. The sequence alignment data also suggests that these clones share some homologue with the C-terminal of the GAP sequence that is known to interact with RAS-protein (7). The DIP1 and DIP2 sequences overlapped, which indicates that both derive from the same cDNA species.

To unveil their expression pattern in different tissues and organs, and to demonstrate each clone's distinct pattern, northern analysis (Fig. 2A in Appendix 2) was performed. For example, DIP7, with a major 6.0 kb transcript, appears to be brain-specific, while DIP1 and DIP2, with at least two transcripts (7.0 kb and 4.7 kb), appear present in brain, kidney, and both prostatic epithelial and stromal cell cultures. This suggests that at least two groups of DIPs are present in brain and other tissues. Interestingly, DIP1/2 was detected in prostatic epithelial cells derived from the basal cells (e.g., NbE and VPE), but it was not detected in intact VP because the basal cell population constitutes only 5% of the total prostatic epithelia in intact VP.

After screening another brain cDNA library, we were able to assemble a full-length cDNA of DIP1/2. As Fig. 2A shows (Appendix 2), the deduced sequence of a full-length DIP1/2 indicates that an open reading of 996 amino acids contains three unique domain: a GAP domain (aa 194-409), a proline-rich domain (aa 727-736), and a leucine zipper domain (aa 842-861). Using synthetic peptide derived from the C-terminus of DIP1/2, we raised a polyclonal antibody specific against DIP1/2 (Fig. 2B in Appendix 2). As Fig. 2C shows (Appendix 2), in contrast to preimmune serum, the polyclonal antibody can specifically recognize a 130 kD protein derived from *in vitro* translation of DIP1/2 cDNA. In the rat brain we observed several additional protein bands that may be produced by protein modification and/or isoform. The polyclonal antibody should be a specific antibody against DIP1/2. Thus far we have used this antibody to determine whether DIP1/2 protein interacts with the C-CAM1 protein.

A similar approach using a yeast two-hybrid system was employed to identify the presence of the downstream effector for the C-CAM1 protein because our laboratory is experienced with this methodology and because this system provides a swift way to clone any cDNA sequence. A bait vector containing the intracellular domain of C-CAM1 is under construction. When it is finished, this bait vector will be subjected to the same screening procedures used in the DOC-2 experiment.

## KEY RESEARCH ACCOMPLISHMENT

- Dissociated the Ig domain (for adhesion activity) of the C-CAM1 protein with its tumor suppression activity.

- Determined the functional domain of the C-CAM1 protein (i.e., intracellular domain) for its tumor suppression activity.

- Demonstrated that there are no direct interaction between C-CAM1 and DOC-2 proteins.

- Identified a novel gene (i.e., DIP1/2) from Ras-GAP family as a downstream effector of DOC-2 protein.

• Cloned a full-length DIP1/2 cDNA and generated a specific antibody for DIP1/2 protein.

• Demonstrated the presence of DIP1/2 in the normal epithelial culture cells derived from basal cell of the rat prostate.

## REPORTABLE OUTCOMES

### FULL-LENGTH PAPER

1.  Hsieh, J.T., Early K, Pong, R.C., Wang, Y., Van, N.T., and Lin S-H. (1999) Structural analysis of C-CAM1 molecule for its tumor suppression function in prostate cancer cells. Prostate, 41, 31-38.

### ABSTRACT

1.  Hsieh, J.T., Tseng, C.P., Wang, Z., Pong, R.C., and Ely, B. (1999) An underlying mechanism of DOC-2 in inhibiting AP-1 activity. Proc. Am. Assoc. Cancer Res., 40, 516.

2.  Tseng, C.P., Wang, Z., Pong, R.C., Li, Y., Ely, B., and Hsieh, J.T. (1999) An underlying mechanism of tumor suppressor gene, DOC-2, in prostate cancer. J. Urol., 160, 60A.

## CONCLUSIONS

The basal cells of the prostate gland, considered as a stem cell population, are not only responsible for maintaining homeostasis of the normal prostate but also contribute to the progression of AI prostate cancer. Until now, the regulatory pathway(s) involved in prostate homeostasis was undefined. In this study we have examined: 1) the functional role of two unique basal cell-associated genes, C-CAM1 and DOC-2, in AI prostate cancer, and 2) the signaling network elicited by these two genes.

We were able to define the intracellular domain containing a Ser/Thr phosphorylation site as a key domain to modulate the tumor suppression function of the C-CAM1 protein (Appendix 1). Our results clearly indicate that the C-CAM1 protein can function like a membrane receptor in order to initiate a cascade of phosphorylation events through a variety of adapter proteins and kinases, which subsequently culminate in a wave of immediate early gene expression in the nucleus. Therefore, because we have ruled out any possible interaction between C-CAM1 and DOC-2 proteins, the immediate study is to identify the effector protein(s) associated with DOC-2 protein.

We have shown that DOC-2 can suppress the *in vitro* growth of prostate cancer cells (5). In our recent study, we demonstrated that phosphorylation of serine 24 in the N-terminus of the DOC-2 protein correlates with its activity (6). Using the yeast two-hybrid screening system we identified a novel gene, DIP1/2, belonging to the Ras-GAP family, as an immediate interactive protein for DOC-2. DIP1/2 appears to express in many tissues, including degenerated prostates and prostatic epithelial cell cultures derived from the basal cell population. Further characterization of the function role of DIP1/2 in signal transduction and the regulation of DIP1/2 by microenvironmental factors will certainly unveil the signal cascade that controls the growth and differentiation of prostatic epithelium.

This study signifies the potential role of homeostatic factors in the progression of prostate cancer. These new molecules may serves as surrogate markers for predicting the biologic behavior of prostate cancer. Moreover, the information gleaned from this study can be used to formulate a new regimen able to intervene sooner during the multiple steps of prostate carcinogenesis.

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Yingming Li, M.B. (Research Assistant)

# APPENDICES

# Structural Analysis of the C-CAM1 Molecule for Its Tumor Suppression Function in Human Prostate Cancer

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**BACKGROUND.** Recently, we demonstrated that expression of C-CAM1, an immunoglobulin (Ig)-like cell adhesion molecule (CAM), was diminished in both prostate intraepithelial neoplasia and cancer lesions, indicating that loss of C-CAM1 expression may be involved in the early events of prostate carcinogenesis. Also, increased C-CAM1 expression can effectively inhibit the growth of prostate cancer. Structurally, C-CAM1 represents a unique CAM with a potential signal transducing capability. In this study, we further analyzed the functional domain of C-CAM1 for controlling its tumor suppression function.
**METHODS.** Recombinant adenoviruses expressing a series of C-CAM1 mutants were generated, such as AdCAMF488 (mutated C-CAM1 containing Tyr-488 → Phe-488), AdCAMH458 (intracellular domain deletion mutant containing 458 amino acids), AdCAMG454 (intracellular domain deletion mutant containing 454 amino acids), and AdCAMΔD1(C-CAM1 mutant containing first Ig domain deletion). After in vitro characterization of each virus, human prostate cancer cells infected with these viruses were subcutaneously injected into athymic mouse. Both tumor incidence and volume were measured for determining the tumor suppression function for each mutant.
**RESULTS.** In vivo tumorigenic assay indicated that AdCAMΔD1 without cell adhesion function still retained its tumor suppression activity. In contrast, both AdCAMH458 and AdCAMG454 decreased or lost their tumor suppression activity.
**CONCLUSIONS.** Our data indicate that the intracellular domain of the C-CAM1 molecule is critical for inhibiting the growth of prostate cancer, suggesting that C-CAM1 interactive protein(s) may dictate prostate carcinogenesis. *Prostate 41:31–38, 1999.*
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KEY WORDS:     cell adhesion molecule; tumor suppressor; prostate cancer

## INTRODUCTION

In multicellular organisms, ontogenesis is orchestrated by cell-cell interactions among different cell types throughout embryogenesis. Cell adhesion molecules (CAMs) play a central role in coordinating the entire process. Very often, altered CAM expression results in changing the homeostasis of normal cells and leads to hyperplastic growth of cells. In recent

studies, we demonstrated that an androgen-repressed CAM [1], C-CAM1, was inversely correlated with the status of premalignant lesions of human prostate cancer, i.e., prostate intraepithelial neoplasia, and cancer lesions as well, indicating that C-CAM1 may be a potent tumor suppressor in prostate carcinogenesis [2]. To demonstrate the tumor-suppressive function of C-CAM1, we transfected a high-tumorigenic prostate cancer line (PC-3) with a C-CAM1 expression vector [3], or infected PC-3 cells with a recombinant adenovirus expressing C-CAM1 cDNA [4]. In both cases, both the in vitro and in vivo growth of prostate cancer cells was significantly inhibited. In addition, by decreasing endogenous expression of C-CAM levels in a nontumorigenic prostatic epithelium cell line with an antisense vector resulted in an increase of the in vivo tumorigenicity of this cell line [3]. Therefore, C-CAM is a potent tumor suppressor in human prostate cancer.

Based on cDNA sequences, the structure of the C-CAM1 molecule is very similar to that of the carcinoembryonic antigen (CEA) and belongs to the immunoglobulin (Ig) gene superfamily. However, C-CAM1 represents a new family of $Ca^{2+}$-independent CAMs because it contains three distinct domains, i.e., the extracellular, the transmembrane, and intracellular domains. The first Ig loop in the extracelluar domain is critical for the intercellular adhesion of C-CAM1 [5]. Also, the transmembrane domain is required for C-CAM1 as a cell surface molecule. Interestingly, the cytoplasmic (or intracellular) domain with 71 amino acids contains at least two potential phosphorylation sites, including one for cAMP-dependent kinase and tyrosine kinase, suggesting that C-CAM1 may function as a receptor to initiate a signaling pathway. However, the functional domain(s) of C-CAM1 as a tumor suppressor in prostate cancer is still unknown. Therefore, we decided to analyze the effect of each domain of the C-CAM1 molecule on the in vivo growth inhibition of prostate cancer.

## MATERIALS AND METHODS

### Construction and Characterization of Recombinant Adenoviruses Containing C-CAM Deletion Mutants

To generate recombinant adenoviruses containing various deletion mutants of C-CAM1 cDNA, the cDNAs were directionally cloned into the HindIII and NotI sites of a shuttle vector (pAdE1CMV/pA) and cotransfected with the pJM17 vector into 293 cells, as described previously [4]. After a large-scale production using two cycles of CsCl ultracentrifugation, the titer of each virus was determined by the plaque

assay as follows: AdCAM101 ($3.4 \times 10^{10}$ pfu/ml); AdCAM902 ($1.1 \times 10^{10}$ pfu/ml); AdCAMΔD1 ($1.8 \times 10^{10}$ pfu/ml); AdCAMF488 ($4.0 \times 10^{10}$ pfu/ml); AdCAMG454 ($3.9 \times 10^{10}$ pfu/ml); and AdCAMH458 ($1.3 \times 10^{10}$ pfu/ml).

In this study, we used both adenoviral DNA and viral infectivity to characterize each recombinant adenovirus. To confirm the presence of the cDNA insert, recombinant adenoviral genomic structure was carried out using the polymerase chain reaction (PCR), as described previously [4]. In the PCR reaction, three sets of primer were used separately: primer set B [6] was used for identifying the presence of the cDNA insert; primer set C [6] was used for identifying the presence of viral sequences; primer set D [5'ATTAC-CGAAGAAATGGCCGC3', 5'CCCATTTAACACGC-CATGCA3'] [7] was used for examining the presence of the E1 region. On the other hand, viral infectivity was determined in the PC-3 cells 24 hr after viral infection by fluorescent-activated cell scanning (FACS) analysis, as described previously [4].

### Determination of Viral Infectivity of PC-3 Cells by Fluorescent-Activated Cell Scanning Analysis

PC-3 cells were infected with different viruses at 100 m.o.i. (multiplicity of infection) and incubated at 37°C for 24 hr. Immunofluorescence staining was carried out as described previously [4], and then the percentage of positive cells was determined by a dual-laser Vantage flow cytometer (Becton Dickinson, Mountain View, CA).

### Determination of C-CAMI Expression and Cell Adhesion Activity in PC-3 Cells by C-CAM Recombinant Adenoviruses

To determine the levels of C-CAM1 expression in viral-infected cells, we performed both Northern and Western blot assays, as described previously [1]. In Northern blot analysis, a radiolabeled C-CAM cDNA fragment generated from BamHI and PstI digestion was used for probing. For determining C-CAM1 protein expression by each clone, cells were infected with virus at 10 m.o.i. for 24 hr. The cell lysate was subjected to Western blot analysis using the antibody specific against C-CAM1 [8,9].

Cell adhesion activity in PC-3 cells after adenoviral infection was carried out for determining the function of the C-CAM molecule. One million PC-3 cells were resuspended in a 1.0-ml medium and mixed gently at room temperature to allow formation of cell aggregation. At any given time, cell adhesion activity was determined reciprocally by the presence of the percentage of single cells counted by a hemacytometer [3].
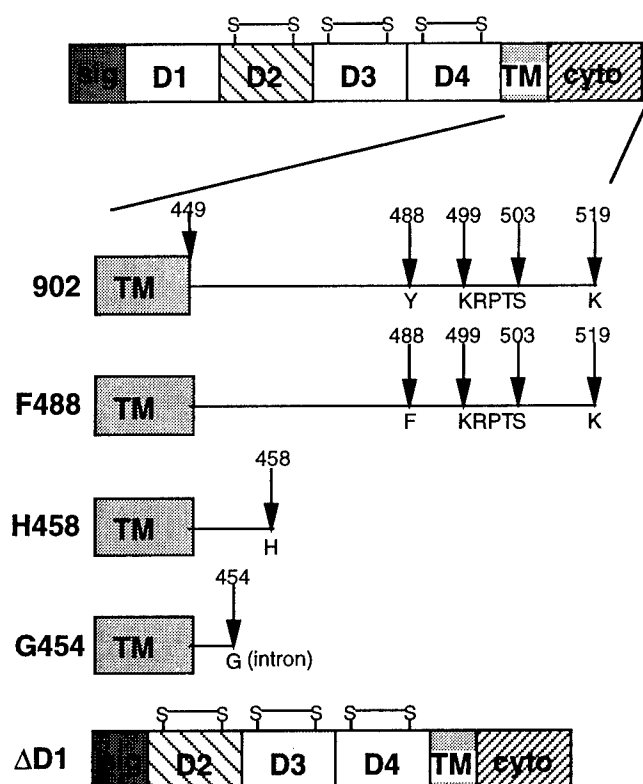
Fig. I. Scheme of C-CAMI structure and its deletion mutants. sig, signal peptide; D1, first Ig domain; D2, second Ig domain; D3, third Ig domain; D4, fourth Ig domain; TM, transmembrane domain; cyto, cytoplasmic domain. Y, tyrosine; K, Lysine; R, arginine; P, proline; T, threomine; S, servine; H, histidine; G, glycine; F, phenylalanine.

## Assessment of In Vivo Tumorigenicity of PC-3 Cells

After viral infection for 18 hr, PC-3 cells were trypsinized, and cell numbers were counted by hemacytometer. One million cells were concentrated in a 100-μl volume and injected subcutaneously at six sites in the flanks of 8–10-week-old male nude mice. Tumors became palpable in about 1 month; the change in tumor volume was measured by a caliper and calculated using the formula described previously [3].

## RESULTS

### Generation and Characterization of the Recombinant Adenoviruses Carrying Various C-CAMI Inserts

To assess the functional domain of C-CAM1 in suppressing tumor growth of human prostate cancer cells, we decided to create a variety of C-CAM1 mutants by altering the potential phosphorylation site or the deleting intracellular domain of the C-CAM1 molecule. As shown in Figure 1, a single base mutation on Tyr-
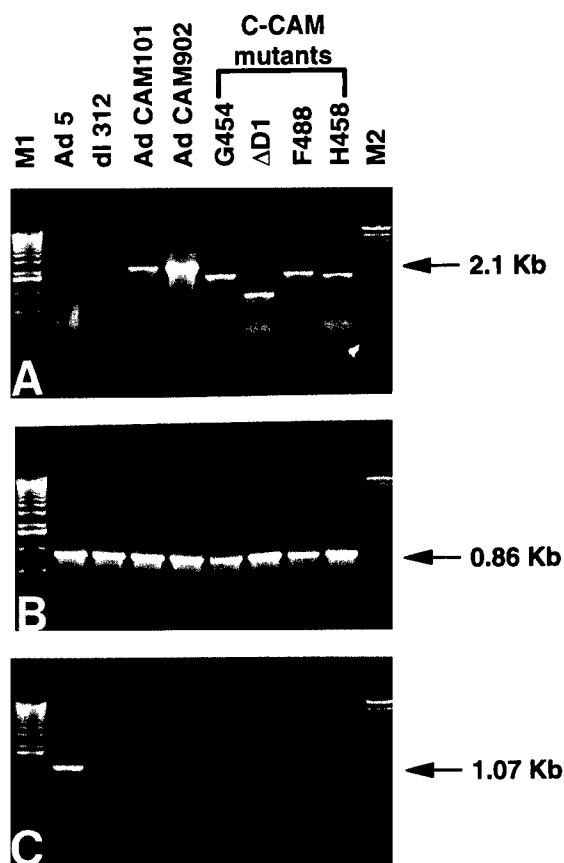


Fig. 2. Characterization of recombinant adenoviruses carrying the various C-CAMI constructs. Recombinant adenoviruses carrying the different C-CAMI deletion constructs were generated from homologous recombination, as described previously [4]. The PCR reaction was carried out for determining the genomic structure of each virus with three different sets of primers: primer set B (A), primer set C (B), and primer set D (C).

488 of C-CAM1 was changed to phenylalanine with site-directed mutagenesis PCR, as described previously [10]. In addition, we deleted most of the amino acids in the intracellular domain to the His-458 position (i.e., AdCAMH458), or removed an additional four amino acids containing a potential Ser/The phosphorylation site to the Gly-454 position (i.e., AdCAMG454). On the other hand, the first Ig domain of C-CAM1 is known to play an important role in cell adhesion function [8]; therefore, a C-CAM1 mutant containing the first Ig domain deletion (i.e., AdCAMΔD1) was created to eliminate its cell adhesion function. Furthermore, two recombinant adenoviruses from a previous study [4], one containing a sense strand of C-CAM1 cDNA (i.e., AdCAM902) and the other containing an antisense strand of C-CAM1 cDNA (i.e., AdCAM101), were used in this study.

We performed a PCR reaction to determine the presence of an individual C-CAM1 insert from each adenovirus. As shown in Figure 2A, the size of each
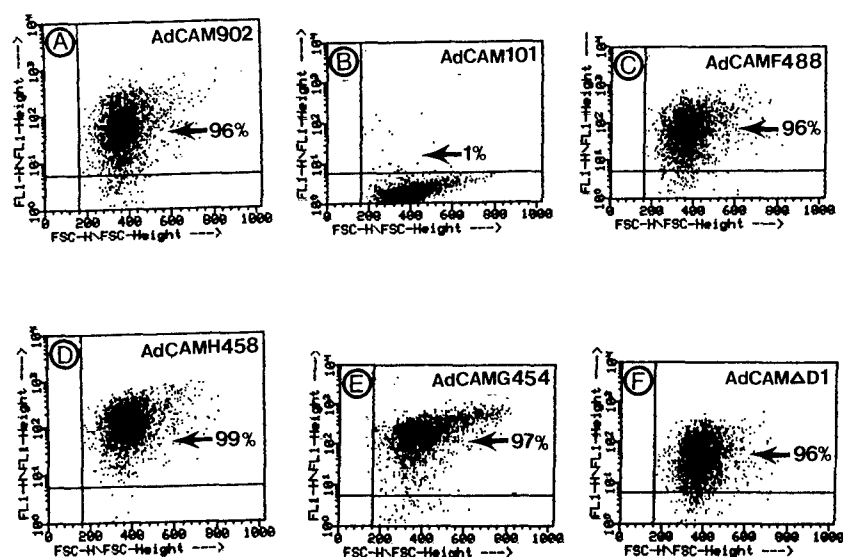
**Fig. 3.** Efficiency of infection by various C-CAMI mutant adenoviruses in PC-3 cells determined by flow cytometry. Cells were infected with different C-CAMI mutant adenoviruses (100 m.o.i.) for 24 hr and were then subjected to fluorescent-activated cell scanning analysis, using immunofluorescence staining. The dual-parameter histogram represents relative cell size (x-axis) measured from forward light scatters (FSC), and log of C-CAMI expression levels (y-axis) measured from the green fluorescence intensity emitted by FITC-stained cells. Positive staining was defined as staining intensity greater than 10 FITC units, which was the background level from control cells stained with antibodies. Numbers in each graph indicate the percentage of positive cells infected by each virus. **A:** AdCAM902. **B:** AdCAM101. **C:** AdCAMF488. **D:** AdCAMH458. **E:** AdCAMG454. **F:** AdCAMΔD1.

PCR product corresponded to that of the individual C-CAM1 mutant. In addition, using a virus-specific primer set (Fig. 2B), we were able to confirm the presence of viral DNA from each recombinant virus. Furthermore, to rule out any possible contamination from wild-type adenovirus in each preparation, we employed a PCR reaction to determine the presence of E1 sequences in each C-CAM1 adenovirus, using the E1-specific primer set. The data in Figure 2C indicate that a 1.07-kb specific band was only detected in the wild-type adenovirus (i.e., Ad5), and not in C-CAM1 viruses and other control viruses such as dl312 with an E1-deletion [11].

Data from the plaque assay indicated that the titer of each virus, ranging from $1 \times 10^{10}$–$4 \times 10^{10}$ pfu/ml, was very similar. Since the plaque assay was determined in 293 cells, it is critical to know whether each virus has an infectivity similar to that of our target PC-3 cells. As shown in Figure 3B, the FACS results demonstrated that no C-CAM1 expression was detected in the AdCAM101-infected cells, because they expressed the antisense C-CAM1 mRNA. In contrast, the numbers of cells infected by each virus at 100 m.o.i. (Fig. 3A,C–F) reached a plateau. Therefore, we decided to use a lower dose of virus to test its tumor suppression function and in order to avoid any artifact due to viral toxicity.

### Determination of Expression and Function of Mutated C-CAMI Protein in PC-3 Cells by Various C-CAMI Adenoviruses

Once these C-CAM1 mutant viruses were generated, we performed both Northern and Western blot analyses to examine the size of C-CAM1 transcript from infected PC-3 cells. PC-3 cells were infected with

different clones of viruses at 10 m.o.i. As shown in Figure 4A, no detectable levels of C-CAM mRNA were found in control PC-3 cells. A full-length C-CAM1 mRNA was detected in PC-3 cells infected with either AdCAM101 or AdCAM902 viruses by a double-stranded cDNA probe. In contrast, a variable-sized C-CAM mRNA transcript was detected in PC-3 cells infected with the rest of the C-CAM1 mutant viruses. Western blot analysis (Fig. 4B) indicated that both AdCAM902 and AdCAMF488 expressed a C-CAM1 protein with 105 kDa. As expected, the molecular weight of mutated C-CAM1 protein induced by each mutant virus was smaller than that of wild-type C-CAM1 protein (Fig. 4B).

The cell adhesive assay was used as a functional test for these deletion mutants. Data in Figure 5 indicate that the control cells and PC-3 cells infected with AdCAM101 did not show any increase in intercellular adhesion. However, cells infected with either AdCAM902 or viruses containing the first Ig domain (such as AdCAMF488, AdCAMH458, and AdCAMG454) showed the same degree of cell adhesion function. In contrast, AdCAMΔD1, a mutant with a deletion of the first Ig domain, failed to elicit any intercellular adhesion in PC-3 cells, indicating that the first Ig domain is critical for the cell adhesive activity of the C-CAM1 molecule.

### Change in Tumor Suppression Activity of C-CAM Mutants

To test the tumor suppression function of each C-CAM1 adenovirus, we infected PC-3 cells with the same titer of viruses overnight. Cells were trypsinized into single-cell suspensions: $1 \times 10^4$ cells were sub-
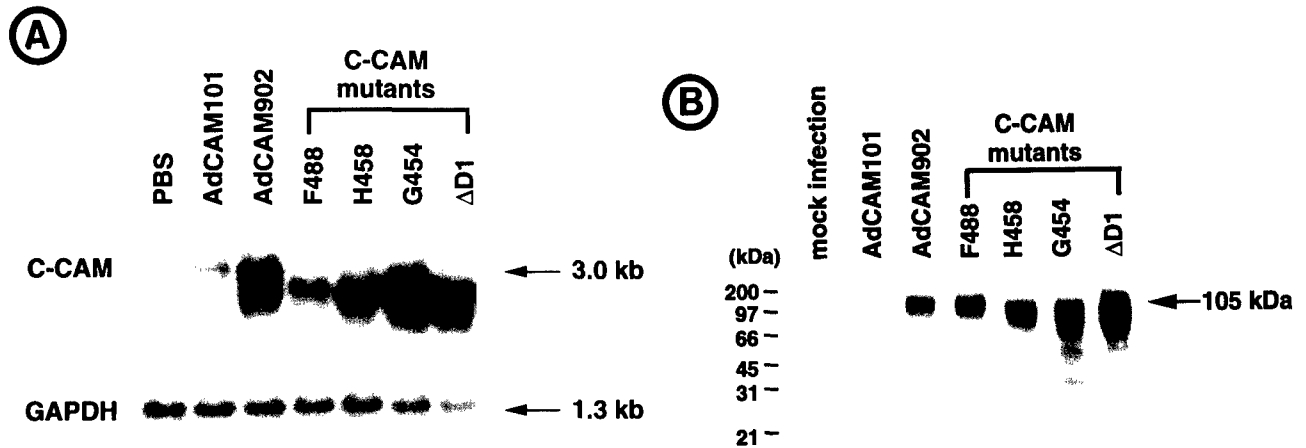
**Fig. 4.** Characterization of C-CAM1 expression in PC-3 cells by infecting the various C-CAM1 adenoviruses. C-CAM1 expression in PC-3 cells infected with the various C-CAM1 viruses was determined by both Northern and Western blot analyses. In Northern blot analysis (**A**), both a random primer radiolabeled C-CAM1 probe [8] and GAPDH as an internal control were used. In Western blot analysis (**B**), Ab669 [2] was used to detect the presence C-CAM1 protein.
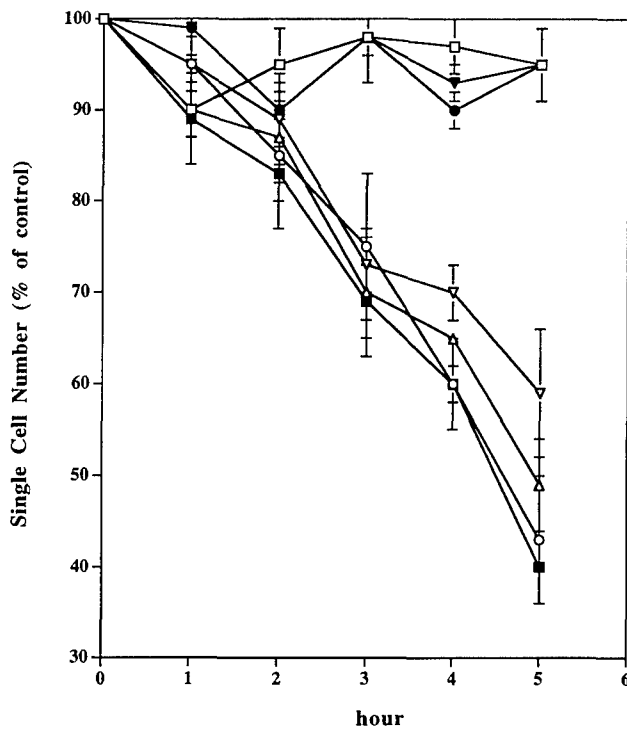


**Fig. 5.** Determination of cell adhesion activity from various C-CAM1 constructs. PC-3 cells were infected with each virus for 24 hr, and then the cells were trypsinized into a single-cell suspension. One million cells from each infection were incubated at room temperature with constant mixing. The percentage of single cells was determined at the times described previously [2] as indicative for the increment in cell adhesion activity. Mock infection (□), AdCAM902 (○), AdCAM101 (●), AdCAMF488 (△), AdCAMH458 (■), AdCAMG454 (▽), AdCAMΔD1 (▼).

jected to FACS analysis for determining the percentage of positive cells prior to injection, and $1 \times 10^6$ cells were injected into the flank of athymic nude mice. Tumors became palpable 5 weeks after injection; tumor incidence and volume were determined during week 8. In Table IA, data indicate that the tumor-suppression effect induced by C-CAM1 adenovirus was dose-dependent. However, at a low dose of virus (5 m.o.i.), we found that the tumor suppression effect was not very significant. At 50 m.o.i., data from both tumor incidence and volume clearly indicate that both the AdCAMF488 and AdCAMΔD1 still retained their tumor suppression function, as observed with AdCAM902. AdCAMΔD1 showed a decrease in its tumor suppression function, which may have been due to the low infectivity evidenced by FACS results (61% positive cells). In contrast, AdCAMH458 and AdCAMG454 decreased or lost their suppression function. AdCAM101, a control virus, showed no tumor suppression effect.

In the second experiment (Table IB), we reduced the viral titer (m.o.i. = 20) to avoid any overdose of viruses. We observed a similar tumor suppression effect from each C-CAM1 mutant virus at an m.o.i. of 20. Data from both experiments demonstrated that AdCAM902, AdCAMF488, and AdCAMΔD1 are potent tumor suppressors. However, the tumor suppression effect induced by AdCAMH458 was intermediate. In contrast, AdCAMG454 completely lost its tumor suppression function. Taken together, these data indicate that the intracellular domain, but not extracellular domain, of C-CAM1 is critical for its tumor suppression function.

**TABLE I. Tumor Incidence and Tumor Volume of PC-3 Cells Inhibited by Either Wild-type C-CAM1 or C-CAM1 Deletion Mutant Adenoviruses**

| A. Treatment[a] | Percentage of positive cells[b] | | Tumor incidence | | Tumor volume (mm$^3$)[c] | |
|---|---|---|---|---|---|---|
| | m.o.i. = 5 | m.o.i. = 50 | m.o.i. = 5 | m.o.i. = 50 | m.o.i. = 5 | m.o.i. = 50 |
| Mock infection | 0 | 0 | 10/12 (83%) | | 95 ± 27 | |
| AdCAM101 | 0 | 0 | 6/6 (100%) | 5/12 (42%) | 105 ± 28 | 69 ± 19 |
| AdCAM902 | 45 | 82 | 8/12 (66%) | 0/12 (0%) | 87 ± 32 | 0 ± 0 |
| AdCAMF488 | 42 | 80 | 12/12 (100%) | 1/12 (8%) | 123 ± 33 | 153[d] |
| AdCAMH458 | 54 | 90 | 8/12 (66%) | 7/12 (58%) | 79 ± 28 | 34 ± 5 |
| AdCAMG454 | 73 | 92 | 10/12 (83%) | 8/12 (66%) | 71 ± 14 | 67 ± 21 |
| AdCAMΔD1 | 39 | 61 | 5/12 (42%) | 6/12 (50%) | 52 ± 20 | 21 ± 3 |

| B. Treatment[a] | Percentage of positive cells,[b] m.o.i. = 20 | Tumor incidence, m.o.i. = 20 | Tumor volume (mm$^3$)[c] m.o.i. = 20 |
|---|---|---|---|
| Mock infection | 0 | 11/12 (92%) | 62 ± 18 |
| AdCAM101 | 0 | 12/12 (100%) | 83 ± 30 |
| AdCAM902 | 77 | 2/12 (17%) | 8 ± 4 |
| AdCAMF488 | 75 | 0/12 (0%) | 0 ± 0 |
| AdCAMH458 | 80 | 6/12 (50%) | 34 ± 12 |
| AdCAMG454 | 82 | 12/12 (100%) | 65 ± 18 |
| AdCAMΔD1 | 82 | 2/12 (17%) | 7 ± 3 |

[a]Mock infection (PBS + 10% glycerol), AdCAM101 (antisense C-CAM1), AdCAM902 (sense C-CAM1 virus), AdCAMF488 (mutated C-CAM1 containing Tyr-488 → Phe-488), AdCAMH458 (C-CAM1 deletion mutant containing 458 amino acids), AdCAMG454 (C-CAM1 deletion mutant containing 454 amino acids), and AdCAMΔD1 (C-CAM1 mutant containing first Ig domain deletion).
[b]Percentage of positive cells were determined by FACS analyses.
[c]Tumor volume was calculated as described previously [3]; number represented mean ± SE.
[d]Only one tumor was observed; therefore, no standard error was calculated.

## DISCUSSION

It is known that CAMs play a central role in coordinating tissue development and epithelial cell differentiation [12–14]. Moreover, altered CAM expression is often associated with carcinogenesis and the metastasis of many neoplasms [15–18]. For example, decreased expression of E-cadherin, a Ca$^{2+}$-dependent epithelial cell-specific CAM, is associated with the progression of several neoplasms [15,19–21]. Recent data from our laboratory and others demonstrated that C-CAM1, a Ca$^{2+}$-independent epithelial cell-specific CAM, can be a potent tumor suppressor in prostate cancer [3,4], colon carcinoma, and hepatocarcinoma [22–24]. Furthermore, a cytogenetic study also showed that deletion of the DCC gene, with a similar Ig-like structure as the C-CAM1, is found in colon cancer [25]. These data indicate that CAMs play a functional role in regulating the carcinogenic process.

C-CAM1 (also named cell-CAM105) is a 105-kDa cell-surface glycoprotein, first detected as the adhesion molecule mediating hepatocyte aggregation [26]. Previously, we observed that the C-CAM1 protein can be detected between cell boundaries of nonpolarized basal prostate epithelial cells in either prolonged cas-

tration prostate [1] or human fetal prostate [2]. These data imply that prostatic epithelial cells may use C-CAM1 as an intercellular adhesion molecule to achieve their cell-cell communication at this stage. This observation is supported by the expression of wild-type C-CAM1 protein in insect cells (i.e., Sf9 cells); using a baculoviral vector system resulted in cell aggregation of Sf9 cells [5]. Upon detailed dissection, of the function of four Ig domains of C-CAM1, Cheung et al. [5] showed that the first Ig domain is required for intercellular adhesion of the C-CAM1 molecule.

In addition to the extracellular domain of C-CAM1, the 71 amino acids of the intracellular domain are also required for the adhesion function of C-CAM1 [27]. Among these amino-acid sequences, there are two consensus sequences for both cAMP-dependent kinase and tyrosine kinase. Also, the putative tyrosine phosphorylation site localized in the antigen-receptor homology (ARH) motif is known to be essential for signal transduction in B-cells [28]. Our recent studies from both natural mutants of C-CAM (e.g., C-CAM3) [27] and deletion mutants generated by site-directed mutagenesis [10] further indicated that the potential Ser/Thr phosphorylation site (amino acids 455–458) in

the intracellular domain, in addition to the first Ig domain, was also critical for C-CAM's cell adhesion function.

Little is known about the functional domain(s) of C-CAM1 in modulating its tumor suppression activity in prostate cancer. Therefore, we decided to generate a variety of deletion mutants ranging from the deletion of the first Ig domain to the deletion of the "potential signal transduction motif" in the intracellular domain of the C-CAM1 molecule. As shown in Table I, both the first Ig domain and the tyrosine phosphorylation site (i.e., amino acid 488) did not play a significant role in modulating the suppression function of C-CAM1 in vivo. Interestingly, H458 lost half of the tumor suppression activity, suggesting that the C-terminal sequences (i.e., the 61 amino acids adjacent to amino acid 458), including ARH domain, may be critical for retaining the tumor suppression function of C-CAM1. Nevertheless, this study indicated that these four amino acids, containing a potential Ser/The phosphorylation site, are crucial for maintaining the tumor suppression function of C-CAM1 (Table I). Similar results were observed in breast cancer cells [29]. Taken together, two domains (amino acids 454–458 and 458–519) of C-CAM1 are crucial for its tumor suppression activity in human prostate cancer. Based on these results, we hypothesized that C-CAM1 protein phosphorylation modulated by protein kinase A may play an important role in suppressing prostate cancer growth. It is likely that the intracellular domain of C-CAM1 may also interact with other soluble factors to transduce its negative signal. An 80-kDa protein was recently identified as a potential interactive protein that is correlated with its growth inhibitory activity [30]. Furthermore, an interaction between C-CAM1 and structural protein may contribute to its biologic function. For example, three cytoplasmic proteins, α-, β-, and γ-catenin, are found to be associated with the cytoplasmic domain of E-cadherin [31]. These associated proteins, which are part of the adherent junction proteins, not only play important roles in maintaining cellular architecture [32], but also have been found to interact with other potential tumor suppressor gene products, e.g., APC [33,34]. Therefore, the potential interactive proteins associated with C-CAM1 warrant further investigation.

In this study, we observed that recombinant adenovirus appears to be an efficient vector to deliver exogenous DNA into target cells (Fig. 2 and Table I) in vitro. Also, our recent results showed that C-CAM1 adenovirus (i.e., AdCAM902) can effectively inhibit in vivo growth of tumors [4], indicating that the C-CAM1 adenovirus is a potent therapeutic agent in prostate cancer. However, because of its wide spectrum of host infectivity [35], recombinant adenovirus may express the transgene in nontarget cells, causing side effects in the host. To alleviate the undesired toxicity elicited by adenoviruses, in the prostate, tissue-specific promoters such as prostate-specific antigen [36] and the probasin gene [37] should be good candidates. Most importantly, this study demonstrated the critical functional domain of C-CAM1 in controlling the in vivo growth of human prostate cancer. Based on these results, C-CAM1 can be further engineered into a "pure" tumor suppressor by removing unnecessary residues, which may increase the therapeutic index for this molecule. Furthermore, identifying the signal pathway elicited by C-CAM1 in prostate cancer can provide a new strategy in fighting this disease.

## ACKNOWLEDGMENTS

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**Fig. 1 The interaction between DOC-2 and DIP1/2.** Cells were cotransfected with both DOC-2 and DIP1/2 expression vectors for 48 h, then cell lysate were immunoprecipitated with either DOC-2 or DIP1/2 specific antibody, then immunocomplex was subjected to western blotting. **1**, cells were cotransfected with both p59, a DOC-2 isoform, and DIP1/2 vectors. **2**, cells were cotransfected with both p82, a DOC-2 isoform, and DIP1/2 vectors. IP: immunoprecipitation. WB, western blotting.
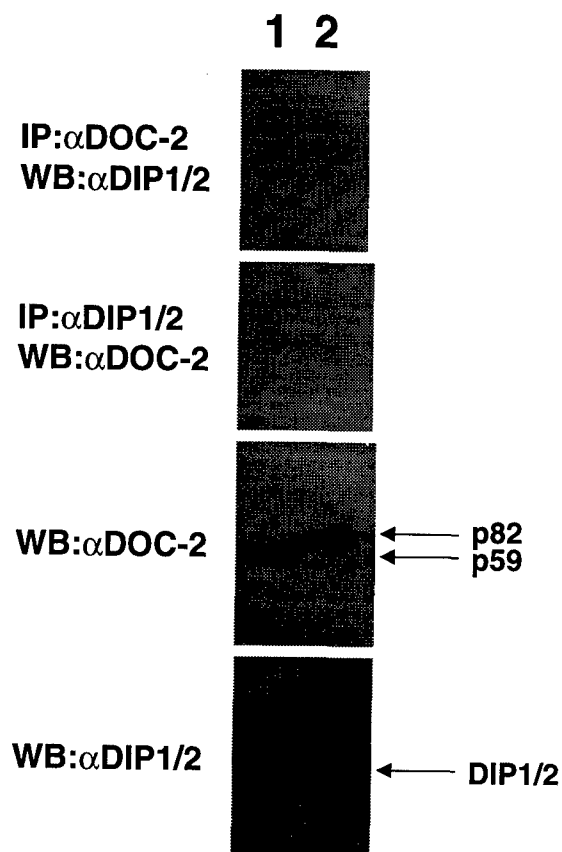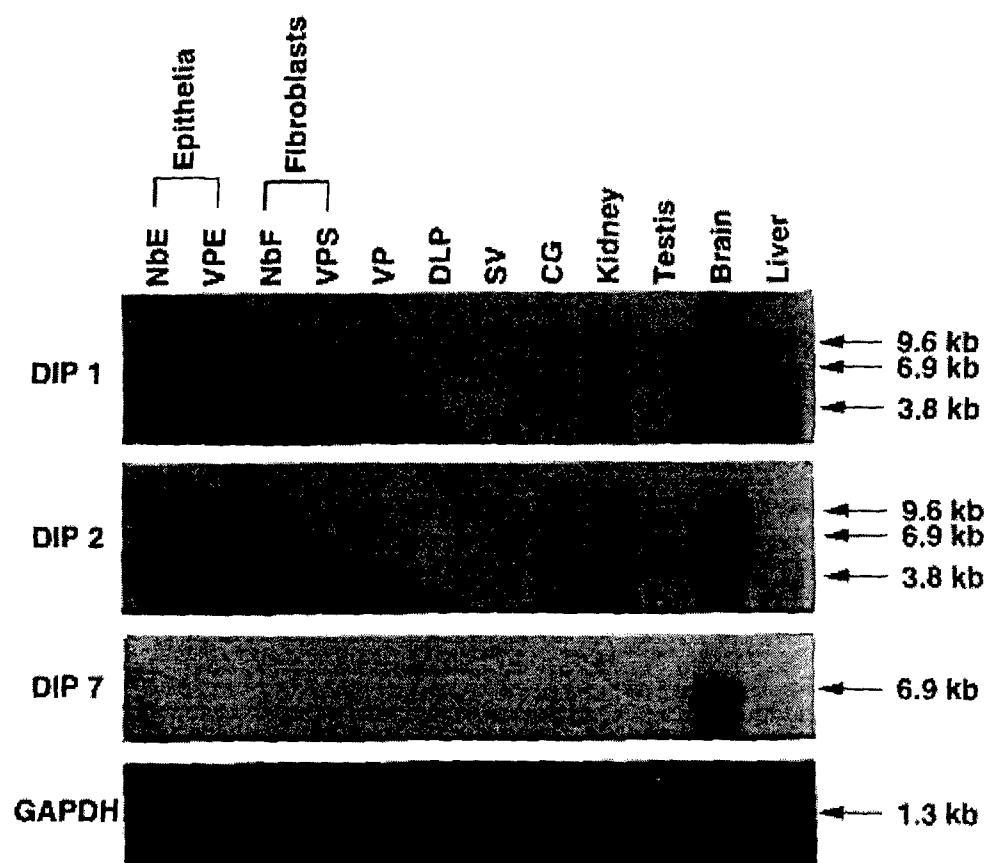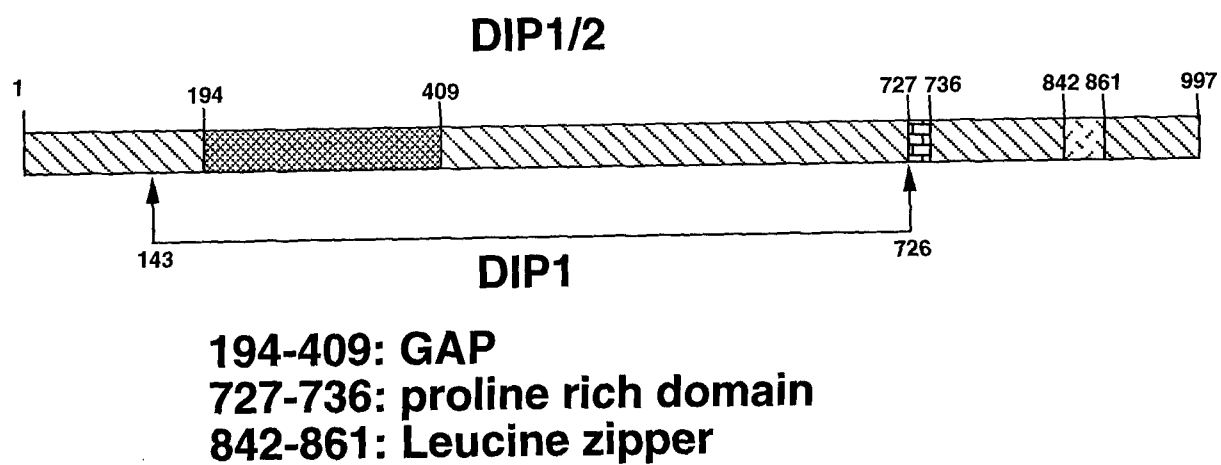


**Fig. 2 Cloning and characterization of DIPs.** A, The differential expression of DIP mRNA in various organs and cell lines. B, The deduced peptide sequence of DIP1/2. NbE and VPE, rat prostatic epithelia; NbF and VPs, VP, rat prostatic stroma, ventral prostate; DLP, dorsalateral prostate; SV, seminal vesicle; CG, coagulating gland. C, Characterization of the polyclonal antibody against DIP1/2. 1, Comparing the antibody recognition between preimmune serum and immune serum. 2, Determining the specificity of the affinity-purified DIP1/2 antibody in the presence of increasing concentrations of peptide competitor. Rat brain extract (20 μg) and *in vitro* translation product of DIP1/2 cDNA were used.
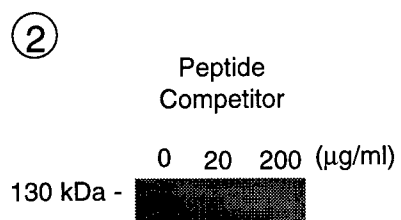
**A**

DIP 1

DIP 2

DIP 7

GAPDH

Epithelia: NbE, VPE
Fibroblasts: NbF, VPS
VP, DLP, SV, CG, Kidney, Testis, Brain, Liver

DIP 1 — 9.6 kb, 6.9 kb, 3.8 kb
DIP 2 — 9.6 kb, 6.9 kb, 3.8 kb
DIP 7 — 6.9 kb
GAPDH — 1.3 kb

**B**

DIP1/2

1    194    409    727  736    842  861    997

143    726

DIP1

194-409: GAP
727-736: proline rich domain
842-861: Leucine zipper

**C**



① Preimmune serum / immune serum

In Vitro DIP1/2 | Rat Brain | In Vitro DIP1/2 | Rat Brain

130 kDa -

② Peptide Competitor

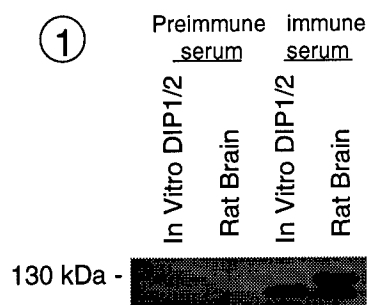0   20   200   (µg/ml)

130 kDa -

# Appendix 3

# The Amino Acid Sequence of DIP1/2

```
MENLRRAVHP NKDNSRRVEH ILKLWVIEAK DLPAKKKYLC CLCLDDVLYA   50
RTTGKLKTDN VFWGEHFEFH NLPPLRTVTV HLYRETDKKK KKERNSYLGL  100
VSLPAASVAG RQFVEKWYPV VTPNPKGGKG PGPMIRIKAR YQTITILPME  150
MYKEFAEHIT NHYLGLCAAL EPILSAKTKE EMASALVHIL QSTGKVKDFL  200
TDLMMSEVDR CGDNEHLIFR ENTLATKGIE EYLKLVGHYL LQDALCEFIK  250
ALYESDENCE VDPSKCSAAD LPEHQGNLKM CCELAFCKII NSYCVFPREL  300
KEVFASWRQE CSSRGRPDIS ERLISASLFL RFLCPAIMSP SLFNLLQEYP  350
DDRTARTLTL IAKVTQNLAN FAKFGSKEEY MSFMNQFLEH EWTNMQRFLL  400
EISNPETLSN TAGFEGYIDL GRELSSLHSL LWEAVSQLDQ SIVSKLGPLP  450
RILRDVHTAL STPGSGQLPG TNDLASTPGS GSSSVSTGLQ KMVIENDLSG  500
LIDFTRLPSP TPENKDLFFV TRSSGVQPSP ARSSSYSEAN EPDLQMANGS  550
KSLSMVDLQD ARTLDGEAGS PVGPEALPAD GQVPATQLVA GWPARAAPVS  600
LAGLATVRRA VPTPTTPGTS EGAPGRPQLL APLSFQNPVY QMAAGLPLSP  650
RGLGDSGSEG HSSLSSHSNS EELAAAAKLG SFSTAAEELA RRPGELARRQ  700
MSLTEKGGQP TVPRQNSAGP QRRIDQPPPP PPPPPPAPRG RTPPTMLSTL  750
QYPRPSSGTL ASASPDWAGP GTRLRQQSSS SKGDSPELKP RALHKQGPSP  800
VSPNALDRTA AWLLTMNAQL LEDEGLGPDP PHRDRLRSKE ELSQAEKDLA  850
VLQDKLRIST KKLEEYETLF KCQEETTQKL VLEYQARLEE GEERLRRQQE  900
DKDVQMKGII SRLMSVEEEL KKDHAEMQAA VDSKQKIIDA QEKRIASLDA  950
ANARLMSALT QLKERYSMRA RNGVSPTNPT KLQITENGEF RNSSNC       996
```

# Sequence Alignment of DIP1/2 with Different RasGAPs

```
DIP       GKVKDFLTDLMMSEVDR-CGDNEHLIFRENTLATKGIEEYLKLVGHKYLQDA
SynGAP    GKAKDFLSDMAMSEVDRFMERE-HLIFRENTLATKAIEEYMRLIGQKYLKDA
rn-GAP    KLESLLLCTLNDREIS--MEDEATTLFRATTLASTLMEQYMKATATQFVHHA
nGAP      GRAKDFLTDLVMSEVDR-CGEHDVLIFRENTIATKSIEEYLKLVGQQYLHDA
hNF1      HLLYQLLWNMFSKEVE--LADSMQTLFRGNSLASKIMTFCFKVYCATYLQKL
p120GAP   KLESLLLCTLNDREISM--EDEATTLFRATTLASTLMEQYMKATATQFVHHA


DIP       LCEFIKALYESDE-----NCEVDPSKCSAAD-LPEHQGNLKMCCELAFCKII
SynGAP    IGEFIRALYESEE-----NCEVDPIKCTAS-SLAEHQANLRMCCELALCKVV
rn-GAP    LKDSILKIMESKQ-----SCELSPSKLEKNEDVNTNLAHLLSILSELVEKIF
nGAP      LGEFIKALYESDE-----NCEVDPSKCSSSE-LIDHQSNLKMCCELAFCKII
hNF1      L-DPLLRIVITSSDWQHVSFEVDPTRLEPSESLEENQRNLLQMTEKFFHAII
p120GAP   LKDSILKIMESKQ-----SECLSPSKLKKNEDVNTNLTHLLNILSELVEKIF


DIP       NSYCVFPRELKEVFASWRQECSSR--GRPDISERLISASLFLRFLCPAIMSP
SynGAP    NSHCVFPRELKEVFASWRLRCAER--GREDIADRLISASLFLRFLCPAIMSP
rn-GAP    MASEILPPTLRYIYGCLQKSVQHKWPTNNTMRTRVVSGFVFLRLICPAILNP
nGAP      NSYCVFPRELKEVFASWKQQCLNR--GKQDISERLISASLFLRFLCPAIMSP
hNF1      SSSSEFPPQLRSVCHCLYQVVSQRFPQNSIG---AVGSAMFLRFINPAIVSP
p120GAP   MASEILPPTLRYIYGCLQKSVQHKWPTMTTMRTRVVSGFVFLRLICPAILNP


DIP       SLFNLLQEYPDDRTARTLTLIAKVTQNLANFAKFGSKEEYMSFMNQFLEHE-
SynGAP    SLFGLMQEYPDEQTSRTLTLIAKVIQNLANFSKFTSKEDFLGFMNEFLELE-
rn-GAP    RMFNIISDSPSPIAARTLTLVAKSVQNLANLVEFGAKEPYMEGVNPFIKSN-
nGAP      SLFNLMQEYPDDRTSRTLTLIAKVIQNLANFAKFGNKEEYMAFMNDFLEHE-
hNF1      YEAGILDKKPPPRIERGLKLMSKILQSIANHVLF-TKEEHMRPFNDFVKSNF
p120GAP   RMFNIISDSPSPIAARTLILVAKSVQNLANLVEFGAKEPYMEGVNPFIKSN-


DIP       WTNMQRFLLEISNPETLS
SynGAP    WGSMQQFLYEISNLDTLT
rn-GAP    KHRMIMFLDELGNVPELP
nGAP      WGGMKRFLLEISNPDTIS
hNF1      DAARRFFLDIASDCPTSD
p120GAP   KHRMIMFLDELGNVPELP
```