The Mechanism of Growth-inhibitory Effect of DOC-2/DAB2 in Prostate Cancer

CHARACTERIZATION OF A NOVEL GTPase-ACTIVATING PROTEIN ASSOCIATED WITH N-TERMINAL DOMAIN OF DOC-2/DAB2*

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DOC-2/DAB2 is a member of the disable gene family with tumor-inhibitory activity. Its down-regulation is associated with several neoplasms, and serine phosphorylation of its N terminus modulates DOC-2/DAB2’s inhibitory effect on AP-1 transcriptional activity. We describe the cloning of DIP1/2, a novel gene that interacts with the N-terminal domain of DOC-2/DAB2. DIP1/2 is a novel GTPase-activating protein containing a Ras GTPase-activating protein homology domain (N terminus) and two other unique domains (i.e. 10 proline repeats and leucine zipper). Interaction between DOC-2/DAB2 and DIP1/2 is detected in normal tissues such as the brain and prostate. Altered expression of these two proteins is often detected in prostate cancer cells. Indeed, the presence of DIP1/2 effectively blocks mitogen-induced gene expression and inhibits the growth of prostate cancer. Thus, DOC-2/DAB2 and DIP1/2 appear to represent a unique negative regulatory complex that maintains cell homeostasis.

EXPERIMENTAL PROCEDURES

Cell Cultures—Three human prostate cancer cell lines (TSU-Pr1, LNCaP, and C4-2) and COS cells were maintained in T medium supplemented with 5% fetal bovine serum (4). PrEC, a primary prostatic epithelial cell derived from a 17-year-old juvenile prostate, was maintained in a chemically defined medium purchased from Clonetics. PZ-HPV-7, a cell line derived from the peripheral zone of a normal prostate (12), was maintained in T medium containing 5% fetal bovine serum. MDAPCa10 and MDAPCa10 cell lines were derived from patients with bony metastasis (13). Three additional primary prostatic epithelial cells, derived from either cancer lesions (SWPC1, SWPC2) or adjacent normal tissue (SWNPC2), were obtained from patients with prostate cancer who had had radical prostatectomy. All these cells were maintained in the same medium as PrEC. Corresponding antibody staining indicated that all primary cells were cytokeratin-positive and vimentin-negative.

Yeast Two-hybrid Screening—Using primers 5′-GAATTCCTCGTCACTTACGAA-3′ and 5′-GGATCTCTACTGAGGCTTTGGTC-3′:: GTPase-activating protein; H, hemagglutinin; PBS, phosphate-buffered saline; EGF, epidermal growth factor; FBS, fetal bovine serum; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; TRE, TPA response element; SRE, serum response element.
DIP1/2, a Novel DOC-2/DAB2-interactive Protein

G-3' and using DOC-2/DAB2 cDNA as a template we generated an 823-bp fragment corresponding to the 5'–end of the cDNA. The PCR-amplified fragment was sequenced before it was subcloned in-frame into pVJL11 vector as a bait construct. Equal amounts of constructed bait vector and pT716 rat brain DNA library vector were co-transfected into SF21 cells. The SF21 uridine–thymidine plates were added to plated on SD-L-T-H (synthetic medium lacking amino acids of leucine, tryptophan, and histidine) plates with 5 μg 3-aminoadenine. Only those colonies that had β-galactosidase activities were further analyzed. Plasmids from those positive clones were rescued and transformed into Escherichia coli HB101 strain for further amplification. Four rounds of phage screening were performed with a rat brain λ ZAP phage library (Stratagene) to clone the full-length cDNA of DIP1/2. After DNA sequencing for each positive clone, the full-length cDNA of DIP1/2 was assembled with the appropriate restriction enzyme digestion.

Northern Blot—Total RNA from various organs of the male rat was isolated with RNAzol (TEL-TEST). Twenty micrograms of total RNA/ lane were separated on 1% formaldehyde agarose gel, transferred onto Zeta-Probe membrane (Bio-Rad), and then hybridized with 32P-labeled DIP1/2 or glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

Generation of the Anti-DIP1/2 Polyclonal Antibody—A peptide sequence (CTNPTKLQITENGEFRNSSNC) corresponding to DIP1/2 amino acid residues 976–996, and with an extra cysteine at the N terminus as a label, was synthesized and used as the antigen to immunize rabbits for generating polyclonal antibody by Zymed Laboratories Inc. After 7 weeks, rabbits were sacrificed to collect antiserum for the antiserum to the coupled SulfoLink gel. After washing with five column volumes of 1M NaCl, they were eluted with elution buffer from the Sulfolink kit (Pierce). Purified antibodies were dialyzed overnight against 4 liters of deionized water at 4°C.

Commmunoprecipitation and Immunoblotting—Cos cells were co-transfected with a series of T7-tagged DOC-2/DAB2 vectors (wild type (p82), alicizing form (p59), N-terminal deletion mutant (ΔN), and C-terminal deletion mutant (ΔB)) and HA-tagged DIP1/2 vector. Cells were lysed with a buffer (50 mM Tris, 5 mM EDTA, pH 8.5; it was then washed with 1 M NaCl). Antibodies against DIP1/2 were first purified by slowly passing the antiserum to the coupled SulfoLink gel. After washing with five column volumes of 1 M NaCl, they were eluted with elution buffer from the Sulfolink kit (Pierce). Purified antibodies were dialyzed overnight against 4 liters of deionized water at 4°C.

RESULTS

Identifying and Cloning DIP cDNA—DOc-2/DAB2's first 280 amino acids were used as a “bait” sequence in the yeast two-hybrid system to search for protein(s) that interacts with the N-terminal domain of DOC-2/DAB2 (17). Of 10,000 transformants screened, 36 positive clones were selected, and two positive clones (DIP1, DIP2) were further analyzed. These two clones shared overlapping sequence and were identical. However, since neither alone contained a full-length sequence, we designated the full-length sequence DIP1/2. To obtain the full-length cDNA of DIP1/2, a λ ZAP cDNA library from a rat brain was screened. Eleven clones spanned about 6.3 kb and represented two different sizes of DIP1/2 mRNA transcripts with different 5' upstream sequences (Fig. 1A). The DIP1/2 cDNA was chosen to have an open reading frame of 996 amino acids and a calculated molecular mass of 110 kDa.

According to the deduced protein sequence, DIP1/2 appears to be a novel protein with several potential functional domains. Its GAP domain with other Ras GAP proteins, its C2 domain with PLCζ, and its SH3 domain with proteins containing an Src homology 3 domain (SH3).

Using LipofectAMINE (Invitrogen), C4-2 cells (2 × 10^5) were transfected with 2 μg of pC1-DIP1/2 or 2 μg of pC1-neo (control). Two days after transfection, cells were selected with G418 (800 μg/ml) and an individual colony was cloned by ring isolation (4). In vitro growth rate of each clone was determined by plating cells in a 24-well plate at a density of 5000 cells/well with T-medium containing 2% TC™ (Celox) and 0.5% FBS. At the indicated days, cell numbers were determined by crystal violet assay (15).

For the colony formation assay, C4-2 cells (3 × 10^4) were plated on a 35-mm dish with T-medium containing 5% FBS and co-transfected with 0.2 μg of β-galactosidase expression vector with 0.8 μg of cDNAs as indicated. Twenty-four hours after transfection, cells were changed to T-medium containing 0.2% FBS. At the indicated time, cells were washed with cold PBS twice and fixed. The number of blue cells was counted by β-galactosidase staining according to Yeung et al. (16).


cDNA—DOc-2/DAB2's first 280 amino acids were used as a “bait” sequence in the yeast two-hybrid system to search for protein(s) that interacts with the N-terminal domain of DOC-2/DAB2 (17). Of 10,000 transformants screened, 36 positive clones were selected, and two positive clones (DIP1, DIP2) were further analyzed. These two clones shared overlapping sequence and were identical. However, since neither alone contained a full-length sequence, we designated the full-length sequence DIP1/2.

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teins (Fig. 1C), including p120 GAP, Homo sapiens neurofibromin (hNF1), synaptic Ras GAP (SynGAP), Rattus norvegicus Ras GAP (rnGAP), and a novel human Ras GAP (nGAP). Boldface letters indicate the consensus amino acid residues within the Ras GAP domain.

Characterizing the DIP1/2 Expression Profile—Northern blot analysis indicated that steady-state levels of DIP1/2 mRNA (about 6.9 kb) are detected in brain, lung, thymus, bladder, and skeletal muscle tissue (Fig. 2A). In both brain and kidney, a different size of RNA transcript with 9.6 kb was found that may represent DIP1/2a with an additional 5′-untranslated sequence (underlined). A, multiple sequence alignment of the GAP domain of DIP1/2 with GAP120, Homo sapiens neurofibromin (hNF1), synaptic Ras GAP (SynGAP), Rattus norvegicus Ras GAP (rnGAP), and a novel human Ras GAP (nGAP). Boldface letters indicate the consensus amino acid residues within the Ras GAP domain.

A polyclonal antibody was raised against a synthetic peptide derived from the C terminus of DIP1/2. With this antibody, a major band of 110 kDa was detected from the in vitrotranslated protein.
scription and translation of DIP1/2 cDNA (Fig. 3A). To further test the specificity of this antibody, serum was incubated with increasing concentrations of synthetic peptide of DIP1/2, ranging from 20 to 200 μg/ml, prior to probing with the blotted membrane. Results (Fig. 3A) indicated that the synthetic peptide competitively binds to the DIP1/2 protein.

The increased protein expression of DIP1/2 was observed in degenerated prostate tissue (Fig. 3B), consistent with elevated DIP1/2 mRNA levels detected in degenerated prostate (Fig. 2B). Since they were parallel with DOC-2/DAB2 levels, these results (Fig. 3B) suggest that both DIP1/2 and DOC-2/DAB2 proteins coexist in degenerated prostate.

To further understand the profile of DIP1/2 and DOC-2/DAB2 expression in human prostate cancer, we screened a variety of prostate cancer cell lines. In Western blot analysis, only a 110-kDa protein band was detected in cell lysates of human prostate cells, indicating that the antibody also recognizes the homologue of human DIP1/2 (hDIP1/2). The sequence of hDIP1/2 is very similar to that of rDIP1/2.2 As shown in Fig. 3C, we found that DIP1/2 and DOC-2/DAB2 proteins were present in both normal primary epithelial cells (PZ-HPV-7, PrEC, and SWNPC2) and primary tumors (SWPC1 and SWPC2). However, a significant decreased expression of DIP1/2 was detected in several metastatic cell lines such as TSU-Pr1, LNCaP, C4-2, MDAPCa2a, and MDAPCa2b, cancer cell lines. We believe this indicates that DIP1/2 is involved in the progression of prostate cancer.

**Specific Interaction between DIP1/2 and DOC-2/DAB2**—Because data from the yeast two-hybrid screening indicated that DIP1/2 and DOC-2/DAB2 directly interact, we further examined whether these two native proteins interact with each other using brain and prostate as tissue sources. Using antibodies against either DOC-2/DAB2 (transfection) or DIP1/2 in a co-immunoprecipitation experiment, we demonstrated that endogenous DIP1/2 and DOC-2/DAB2 proteins were present in the same immune complex (Fig. 3D). Noticeably, there were two sizes of DIP1/2 protein in the rat brain (molecular masses of 110 and 135 kDa, respectively) (Fig. 3D), and the predominant protein appears to be 135 kDa, which may correspond to the 9.6 kb of DIP1/2 mRNA detected in brain tissue (Fig. 2A).

To confirm whether DIP1/2 only interacts with the N-termi-

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Function of DIP1/2 in Vitro and in Vivo—Due to the high sequence homology between DIP1/2 and other Ras GAPs, we thought it likely that DIP1/2 facilitates Ras GTPase activity. To test this, we prepared a GST-DIP1/2 fusion protein containing the minimal Ras GAP domain (23), and either this fusion protein or GST protein was incubated with human recombinant \( \gamma - \text{Ras} \)-bound Ha-Ras protein. The increasing amounts of GST-DIP1/2 (ranging from 0.2 to 1 \( \mu \)g) stimulated Ras GTPase activity in a dose-dependent manner (Fig. 5A). Conversely, DIP1/2 protein (1 \( \mu \)g) had no effect on Ras GTPase activity.

To further compare the Ras GAP activity of DIP1/2 with a known Ras GAP protein (GAP120), we created a DIP1/2 cDNA construct (DIP1/2\textsubscript{m}) as a control with a point mutation (R220L) that disrupts GAP activity. As shown in Fig. 5B, the overall GAP activity between p120\textsuperscript{GAP} and DIP1/2 is very similar, and the DIP1/2\textsubscript{m} did not have any GAP activity toward Ha-Ras. These data clearly demonstrate that DIP1/2 can stimulate GTPase activity of Ha-Ras in vitro. To examine the specificity of DIP1/2 to other small G-proteins such as K-Ras, R-Ras, TC21, and Rap1A, we found that DIP1/2 has a similar GAP activity as p120\textsuperscript{GAP} by stimulating GTPase activity of K-Ras, R-Ras, and TC21 but not Rap1A (Fig. 5C). These data indicate that DIP1/2 is a typical Ras GAP.

Early study of Ras signal transduction indicates that Raf is an immediate downstream effector for Ras signaling (24). Because Raf binds tightly to the GTP-bound form of Ras but not to the GDP-bound form, such differential affinity can be used to determine the GTP-bound status of Ras. To analyze the GAP activity of DIP1/2 in vivo, C4-2 cells (25) were transfected with vectors expressing HA-tagged Ras, DIP1/2, or DIP1/2\textsubscript{m}. After activating Ras using EGF, the GTP-bound form of Ras was precipitated with GST-RBD (GST-Raf containing Ras binding domain). Precipitated Ras was detected using the HA-antibody. As shown in Fig. 5D, in the presence of EGF, the amount of GAP-bound Ras increased over that of the control. Levels of the GTP-bound Ras significantly decreased in cells expressing DIP1/2; however, DIP1/2\textsubscript{m} failed to stimulate Ras GTPase in cells treated with EGF. The whole cell lysates were examined for expression of DIP1/2 and Ras proteins, and results demonstrated that expressions of DIP1/2 and Ras were identical between each transfection. These results indicate that DIP1/2 can function as a Ras GTPase-activating protein in vivo. Therefore, we conclude that DIP1/2 functions as a Ras GAP in vivo and in vitro.

Regulation of the Ras-Raf Signaling Pathway by DIP1/2—The Ras protein functions as an essential component in many intracellular signaling pathways responsible for differentiation, proliferation, and apoptosis (26). The Raf-MEK-ERK pathway is a key signal transduction pathway modulated by Ras protein (27). The downstream components of this pathway, including AP-1, which binds to TRE, and EIK-1, which binds to SRE, subsequently activate gene expression (28–30). Since PKC is able to activate the Raf/MEK/ERK axis (31, 32), we investigated the impact of DIP1/2 on this cascade. As shown in Fig. 6A, in the absence of EGF, increased expression of DIP1/2 could inhibit the basal levels of SRE reporter gene activity in prostate cancer cells.

The presence of EGF increased the reporter gene activity at least 5-fold. However, DIP1/2 could inhibit the reporter gene activity in a dose-dependent manner. Using the same reporter gene assay, we found that DIP1/2 or \( \Delta B \), a DOC-2/DAB2 cDNA

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**Fig. 4.** Direct interaction between DIP1/2 and DOC-2/DAB2 or DAB1. COS cells were cotransfected with different T7-tagged DOC-2/DAB2 constructs and HA-tagged DIP1/2 constructs for 48 h. The supernatants were immunoprecipitated with either T7-antibody-conjugated agarose beads (A) or HA-antibody plus protein A/G-agarose beads (B). After centrifugation, pellets were subjected to immunoblotting analysis. The levels of protein expression from each transfection were determined by Western analysis (C) and (D). Cell lysate was prepared from COS cells transfected with each expression vector and subjected to pull-down by glutathione beads and then probed with DIP1/2 antibody (E). IP, immunoprecipitation; IB, immunoblotting.
containing the N-terminal domain (11), alone can suppress SRE activity (Fig. 6B), but co-expression of ΔB and DIP1/2 had an additive effect on the inhibition of SRE activity in the presence of TPA. These data indicate that physical interaction between DIP1/2 and DOC-2/DAB2 has functional impact on Ras-mediated signal transduction.

Previously, we demonstrated that PKC-elicited DOC-2/DAB2 phosphorylation can block TPA-induced gene activity (11). Therefore, we investigated whether DIP1/2 is a mediator involved in this action. We employed the C4-2 cell line because both DOC-2/DAB2 and DIP1/2 are not detectable. Either a high concentration of ΔB or DIP1/2 alone was able to inhibit TPA-induced TRE reporter gene activity (Table I). At this concentration, we observed only an additive effect on inhibiting TRE reporter gene activity in the presence of ΔB and DIP1/2. When the concentration of ΔB or DIP1/2 decreased to 0.1 or 0.2 µg, respectively, we noticed that the inhibitory effect of each individual cDNA reduced significantly. Transfecting both cDNAs at this concentration, we observed a synergistic inhibitory effect on C4-2 cells. On the other hand, combing the ΔB-S24A mutant with DIP1/2 failed to have any synergistic effect; it appears that Ser24, a PKC substrate, in DOC-2/DAB2 is the key amino acid to modulate this activity. Thus, the binding of DIP1/2 to ΔB can be enhanced by TPA, whereas ΔB-S24A cannot (Fig. 6C). Taken together, these data indicate that the interaction between DIP1/2 and the N-terminal domain of DOC-2/DAB2 has a significant functional impact on gene transcription.

**Biological Effect of DIP1/2 on Prostate Cancer Cells**—Because DIP1/2 appears to be a negative regulator for the Ras-mediated pathway, it may function as a growth inhibitor. To test this, C4-2 cells (a tumorigenic human prostate cancer cell line) were transfected with a DIP1/2 expression vector. Initially, we observed that there were fewer surviving clones in the DIP1/2-transfected plate than in plasmid control-transfected cells despite the same number of cells being used in transfection. After isolating two independent colonies (D1, D2), the protein levels of DIP1/2 in the D2 subline were higher than those in the D1 subline (Fig. 7A). Data from Fig. 7B indicated that expression of DIP1/2 significantly inhibited the in vitro cell growth compared with the plasmid control. This inhibitory effect of both D1 and D2 correlated with their DIP1/2 protein levels (Fig. 7A).

To rule out possible artifacts from stable transfection, we examined the growth suppression of DIP1/2 in C4-2 cells using transient transfection (16). As shown in Fig. 7C, the elevated DIP1/2 expression, determined by Western blot, inhibited the colony formation of C4-2 cells in a time-dependent manner.

![Fig. 5. In vitro and in vivo Ras GAP activity assays. A, kinetics of Ras GAP activity of DIP1/2 protein. One microgram of purified GST protein and different amounts of GST-DIP1/2 were incubated with](image)
We also examined whether the growth suppression effect of DIP1/2, a Novel DOC-2/DAB2-interactive Protein

C4-2 cells can be enhanced in the presence of both DIP1/2 and DOC-2/DAB2. In this experiment, we used half of the amount of DIP1/2 cDNA from the previous experiment (Fig. 7C) and only the N-terminal domain of DOC-2/DAB2 (i.e., ΔB), since we found some additional inhibitory activity associated with the C terminus (10). As shown in Fig. 7D, 0.1 μg of ΔB alone did not exhibit any growth-inhibitory effect, and DIP1/2 alone had a growth-inhibitory effect. However, we observed a synergistic effect of growth suppression of C4-2 cells transfected with both DIP1/2 and ΔB cDNAs compared with C4-2 cells transfected with either DIP1/2 or ΔB alone (Fig. 7D). In contrast, C4-2 cells transfected with both DIP1/2 and ΔB did not exhibit enhanced growth suppression. These data indicated that the interaction between DIP1/2 and DOC-2/DAB2 is critical for the growth-inhibitory effect of DIP1/2 in prostate cancer cells.

**DISCUSSION**

The DIP1/2 expression profile in different organs appears to be diverse. Northern analysis (Fig. 2A) indicates a high level of DIP1/2 mRNA expression in brain, thymus, and bladder tissue and a low level in skeleton muscles, kidney, and liver tissue. But no expression can be detected in several urogenital organs, including the ventral prostate, dorsolateral prostate, seminal vesicle, and coagulating gland. This unique pattern of tissue distribution implies that DIP1/2 may have a specific physiological function in each organ. For example, DIP1/2 expression is detected in the enriched basal cell population of degenerated prostate and in prostatic epithelial cells lines (such as NbE and VPE) derived from the basal cell population (Fig. 2A), suggesting that DIP1/2 may be involved in prostate regeneration. This hypothesis can be supported by our results: 1) decrease or absence of either DIP1/2 or DOC-2/DAB2 is often detected in several metastatic human prostate cancer cell lines (Fig. 3C), and 2) DIP1/2 appears to be a potent growth inhibitor for human prostate cancer cells (Fig. 7). It is known that increased Ras activity is associated with high grade metastatic prostate cancer; however, RAS mutation is rarely detected (33, 34). Our results suggest an underlying mechanism with which to account for this phenomenon. In addition to DIP1/2, we found that altered expression of p120GAP is associated with prostate cancer cells (data not shown). Thus, our results indicate that altered Ras GAP expression plays a critical role in the progression of prostate cancer.

The phosphorylation of Ser24 in DOC-2/DAB2, which is involved in inhibiting TPA-induced AP-1 activity (11), provides evidence for the underlying functional mechanism of DOC-2/DAB2. In this study, our data indicate that DIP1/2 is an immediate downstream effector for DOC-2/DAB2 in both prostate and brain tissues (Fig. 3D), and the binding of DIP1/2 to DOC-

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**TABLE I**

| Relative luciferase activity in prostate cancer | Experiment I | Experiment II |
|-----------------------------------------------|-------------|-------------|
| **Relative luciferase activity**               | PCI-neo     | ΔB          |
| Fold                                          | 33.0 ± 0.2  | 38.3 ± 0.7  |
| S.D.                                          | (100%)      | (100%)      |
| **ΔB**                                        | 13.7 ± 1.9  | 37.0 ± 0.9  |
| **ΔB-S24A**                                   | 33.1 ± 0.3  | 37.1 ± 1.9  |
| **DIP1/2**                                    | 12.4 ± 1.3  | 23.8 ± 1.7  |
| **ΔB + DIP1/2**                               | 6.3 ± 0.2   | 7.3 ± 0.5   |
| **ΔB-S24A + DIP1/2**                          | 11.4 ± 2.1  | 24.4 ± 1.1  |

*The relative luciferase activity is expressed a -fold induction compared with ethanol control. Both experiments I and II are typical representations from several experiments. In experiment I, 0.4 μg of each cDNA was used. In experiment II, ΔB or ΔB-S24A (0.1 μg) and DIP1/2 (0.2 μg) were used. The results were calculated as mean ± S.D. in triplicate.

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Conversely, increased DIP1/2 expression did not effect colony formation of C4-2. These data indicate that DIP1/2’s GAP activity modulates its growth-inhibitory effect. DIP1/2 alone appears to be a potent growth inhibitor for prostate cancer.

We also examined whether the growth suppression effect of
FIG. 7. **Growth-inhibitory effect of DIP1/2 on prostate cancer cells.** Cells were transfected with either pCI-neo or DIP1/2 expression vector. After G418 selecting, two independent clones were isolated and characterized. A, increased protein expression was detected by DIP1/2 antibody. B, the *in vitro* growth rate for each clone was determined by crystal violet assay. C, the colony formation of cells transfected with DIP1/2 cDNA. D, the colony formation of cells transfected with DIP1/2 cDNA and/or DOC-2/DAB2 cDNA. Data represent the mean ± S.D. from six determinants. *, significantly different from plasmid control (p < 0.01).
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2/DAB2 can be enhanced when the Ser24 residue in DOC-2/DAB2 is phosphorylated (Fig. 6C). The most conserved region in DIP1/2 protein is the GAP domain, which has a high amino acid sequence homology (40–90%) compared with the GAP domains of other Ras GAPS, and DIP1/2's GAP domain contains all 31 consensus amino acids of other Ras GAPS. These consensus amino acid residues in the Ras GAP domain modulate Ras GTPase activity (13). For example, Arg220 of p120GAP participates in catalysis and simultaneously stabilizes GTP of Ras for optimal GTP hydrolysis (35). Our data (Fig. 5) indicate that DIP1/2 has Ras GAP activity in vitro and in vivo. Since Arg220 of DIP1/2 is equivalent to Arg220 of p120GAP, once Arg220 was altered (R220L), the single amino acid mutant of DIP1/2 lost its Ras GAP activity in vitro and in vivo (Fig. 5, B and D). Similar to the GAP activity of p120GAP (36), DIP1/2 can stimulate the GTPase activity of several small G-protein of Ras family (Fig. 5C). In majority of PCa cell lines used in this study, we were able to detect the presence of R-Ras, K-Ras and Ha-Ras (data not shown). Therefore, we believe that loss of GAP protein in prostate cells may be an underlying mechanism leading to constitutive activation of Ras in these cells.

We demonstrate that co-expression of DIP1/2 and the N-terminal domain of DOC-2/DAB2 (i.e. DB) has an additive effect on suppressing either TPA-induced SRE or TRE reporter gene activity (Fig. 6B and Table I). Therefore, we believe that the interaction of DIP1/2 with the N-terminal domain of DOC-2/DAB2 acts as a feedback mechanism to modulate PKC-induced gene activation. It is known that the modulation of the Raf/MAPK/ERK axis is also controlled by growth factors through their protein receptor tyrosine kinase, although our data indicate that DIP1/2 alone is also able to inhibit EGF-induced SRE reporter gene activity (Fig. 6A) and cell growth. However, the interaction between DIP1/2 and DOC-2/DAB2 certainly amplifies their individual inhibitory effect (Figs. 6C and 7D and Table I). Therefore, this protein complex containing both DOC-2/DAB2 and DIP1/2 represents a unique negative regulatory machinery to balance signals elicited by growth factors (such as EGF) or PKC activators (such as TPA).

In addition to the N-terminal domain of Ras GAP homology domain of DIP1/2, the proline-rich repeats and leucine zipper domains from its C terminus may contribute to DIP1/2 activity. Our preliminary data indicate that the proline-rich repeats (residues 727–736) in DIP1/2 interact with Grb2 (data not shown). Because Grb2 binds to SOS, a guanine nucleotide exchange factor critical for downstream signaling, the binding of DIP1/2 to Grb2 may interrupt Ras activation. It is also possible that DIP1/2 can interact with other proteins containing the Src homology 3 domain. On the other hand, the leucine zipper domain (residues 842–861) of DIP1/2 may form a homodimer or a heterodimer with other proteins. Although no direct evidence has been shown for DIP1/2 dimerization, we observed a self-dimerization of DIP1/2 using the yeast two-hybrid experiment, which suggests that the dimerization of DIP1/2 may affect its activity. More detailed studies are under way to examine this hypothesis.

In summary, both DIP1/2 and DOC-2/DAB2 form a unique protein complex (Fig. 8) with a negative regulatory activity that modulates the Ras-mediated signaling pathway. This complex is operative in basal cells of the prostate and may orchestrate the differentiation and proliferation potential of these cells during prostate regeneration. In contrast, altered expression of any component of this complex may result in abnormal growth and/or the acquired malignant phenotypes of prostate cancer and perhaps other types of cancer such as ovarian and breast cancer. Further dissection and functional examination of each component in this complex is warranted.

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