The Role of DOC-2/DAB2 Protein Phosphorylation in the Inhibition of AP-1 Activity

AN UNDERLYING MECHANISM OF ITS TUMOR-SUPPRESSIVE FUNCTION IN PROSTATE CANCER

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Ching-Ping Tseng, Brent D. Ely, Rey-Chen Pong, Zhi Wang, Jian Zhou, and Jer-Tsong Hsieh
From the Department of Urology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9110

DOC-2/DAB2, a novel phosphoprotein with signal-transducing capability, inhibits human prostatic cancer cells (Tseng, C.-P., Ely, B. D., Li, Y., Pong, R.-C., and Hsieh, J.-T. (1998) Endocrinology 139, 3542-3553). However, its mechanism of action is not understood completely. This study delineates the functional significance of DOC-2/DAB2 protein phosphorylation and demonstrates that in vivo activation of protein kinase C (PKC) by 12-O-tetradecanoylphorbol-13-acetate (TPA) induces DOC-2/DAB2 phosphorylation, including a serine residue at position 24. Mutation of Ser24 to Ala reduced DOC-2/DAB2 phosphorylation by PKC. Using a synthetic Ser24 peptide (APSKKEKKKSEKTD) or recombinant DOC-2/DAB2 as substrates, PKCβII, PKCγ, and PKCδ (but not casein kinase II) directly phosphorylated Ser24 in vitro. This indicates that DOC-2/DAB2 is a PKC-specific substrate. Since expression of wild-type DOC-2/DAB2, but not the S24A mutant, inhibited TPA-induced AP-1 activity in prostatic epithelial cells, phosphorylation of Ser24 appears to play a critical role in modulating TPA-induced AP-1 activity. Taken together, these data suggest that PKC-regulated phosphorylation of DOC-2/DAB2 protein may help its growth inhibitory function.

DOC-2 (deletion in ovarian carcinoma 2), with a molecular mass of 82 kDa, belongs to the Disabled (Dab) gene family. Recently, we demonstrated that DOC-2/DAB2 is up-regulated in degenerated rat ventral prostate induced by androgen deprivation (1). Histologically, elevated levels of DOC-2/DAB2 are associated with an enriched basal cell compartment, a progenitor cell for glandular epithelium. This suggests that DOC-2/DAB2 may be involved in the homeostasis of prostate regeneration (1). In addition, stable expression of DOC-2/DAB2 in a prostatic cancer cell line significantly reduces its in vivo growth rate concomitant with an increase in G1 cell fractions. It also decreases anchorage-independent growth on soft agar (1). Other types of cancer cell lines, such as the SKOV3 ovarian cancer cell line (2) and the choriocarcinoma cell lines Jar, JEG, and BeWo (3), demonstrate similar results. Therefore, DOC-2/DAB2 appears to be a potent negative regulator of carcinoma cell growth.

The primary structure of DOC-2/DAB2 reveals that DOC-2/DAB2 is a putative signaling molecule with protein-protein interaction and protein phosphorylation as two possible mechanisms modulating its activity (1). The N-terminal phosphotyrosine-interacting domain shares significant homology with mouse DAB1 (4). Disruption of the mouse Dab1 gene disturbs neuronal layering in the cerebral cortex, hippocampus, and cerebellum (5). The similar phenotypes of mouse Dab1 null mice, Reeler, Scrambler, and Yotari, indicate that mouse DAB1 functions as a signaling molecule regulating cell positioning in the developing brain (6). Indeed, the phosphotyrosine-interacting domain of mouse DAB1 is found to bind SRC protein-tyrosine kinase, and therefore, mouse DAB1 may play a key role in key signal transduction pathways involved in the formation of neural networks (4). In addition to the phosphotyrosine-interacting domain, DOC-2/DAB2 also contains a C-terminal proline-rich domain that interacts with the SH3 domain of GRB2 and reduces the binding between GRB2 and SOS. This suggests that DOC-2/DAB2 may modulate growth factor/Ras pathways (7).

DOC-2/DAB2 contains several consensus protein kinase C (PKC), casein kinase II (CKII), and cAMP-dependent protein kinase phosphorylation sites (1), implying that protein phosphorylation may modulate DOC-2/DAB2 activity. Colony-stimulating factor-1 induces DOC-2/DAB2 protein phosphorylation in a mouse macrophage cell line (8). We also found that the phosphorylation of DOC-2/DAB2 is modulated by receptor protein-tyrosine kinase pathways, such as the epidermal growth factor, and the PKC activation pathway, such as stimulation by 12-O-tetradecanoylphorbol-13-acetate (TPA) as described in this study. PKC is the receptor of TPA and is a member of the serine/threonine kinase family composed of at least 12 isoforms (9). Structural and functional studies indicate that PKC isoforms are likely to have distinct and distinguishable functions (10-15).

One of the properties ascribed to activation of PKC by TPA is the ability to alter gene expression. Among genes transcriptionally induced by TPA are ornithine decarboxylase, collagenase, and stromelysin. The promoter regions of several TPA-inducible genes share a conserved TPA-responsive element recognized by the transcription factor AP-1 (16). This study investigated the impact of DOC-2/DAB2 protein phosphorylation on the PKC-mediated signal transduction cascade. We have mapped the TPA-induced DOC-2/DAB2 protein phosphorylation site to Ser24, which appears to modulate the DOC-2/DAB2 inhibition of AP-1 transcription activity. Results indicate that phosphorylation of Ser24 is mediated by PKCβII, PKCγ, and PKCδ, but not CKII. This suggests that the PKC phosphorylation of Ser24 in DOC-2/DAB2 may be an underlying mechanisms for its tumor-suppressive function.

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‡ To whom correspondence should be addressed. Tel.: 214-648-3988; Fax: 214-648-8786; E-mail: hsieh@utsw.swmed.edu.

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**EXPERIMENTAL PROCEDURES**

**Materials**—The AP-1 reporter gene construct −73/+63-Col-luc was provided by Dr. Michael Karin (University of California, San Diego, CA). The T7-tagged pRSV-PKCζβ1, pRSV-PKCγ and pRSV-PKCδ expression plasmids have been described previously (17). The Serα peptide (APSA KK KKKKGGSEKTD) and the Alaα peptide (APAαKK KKKKGGSEKTD) were synthesized by Genemed Biotechnologies, Inc. (San Francisco, CA).

**Cell Cultures**—COS, NbE, and C4-2 cells were maintained in T medium supplemented with 5% fetal bovine serum as described previously (1).

**Construction of Plasmids**—The Salt-NoI fragments of pCl-neo-p82 and pCl-neo-p59 (1) were subcloned into pET-21b(+); and the XbaI-NoI fragments of pET-21b(+)-p82, and pET-21b(+)-p59, containing p82 and p59 cDNA, respectively, were subcloned back into the pCl-neo vector to make the T7-p82 and T7-p59 expression plasmids. The ΔB mutant was created by deleting a 1.2-kilobase Bsu36I fragment from pCl-T7-p82. The ΔN mutant was generated by four-fragment ligation with appropriate restriction enzymes, resulting in the deletion of the first 636 base pairs of p82. Site-directed mutagenesis with polymerase chain reaction was used to create ΔB-S24A, ΔB-S24A, ΔB-S24A, S24A, and ΔB-S24A, S24A, S24A. The BsaI fragments of both p82 and p59 were subcloned into BsaBI-digested ΔB-S24A to create a single amino acid mutant of both p82 and p59.

**[32P]Orthophosphate Cell Labeling**—Metabolic labeling of cells was performed as described (18), and immunoprecipitated, [32P]labeled DOC-2/DAB2 was subjected to phosphoamino acid analysis (19) or cyanogen bromide phosphopeptide mapping (20).

**In Vitro Protein Kinase Assay**—The immunocomplex PKC assay was performed as described (21) in the presence or absence of the activators. The CKII protein kinase assay was performed as described by the manufacturer (Promega, Madison, WI) with equal molar concentrations of the indicated substrates.

**Report Gene Assay**—C4-2 cells (3 × 10⁷ cells/35-mm plate) were transfected with an equal amount of DNA mixture. In every experiment, 0.38 μg of −73/+63-Col-luc and 0.38 μg of pRSV-β-galactosidase plus the indicated amount of DOC-2/DAB2 expression plasmid was included. To ensure that an equal amount of DNA mixture was added to each dish, pCl-neo (control plasmid) was used to make up the differences between each condition. Luciferase and β-galactosidase activities were determined as described (21). Fold induction = ([TPA(luciferase activity (treatment − background))/β-galactosidase activity (treatment − background)])/[ethanol(luciferase activity (treatment − background))/β-galactosidase activity (treatment − background)].

**Immunoprecipitation Assay**—The recombinant GRB2-GST fusion protein was induced by isopropyl-β-D-thiogalactopyranoside for 4–6 h. The bacteria were spun down, and lysed in phosphate-buffered saline supplemented with 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The lysate was sonicated, and then all insoluble material was spun down. The GRB2-GST protein was immobilized on glutathione-Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's instruction. The supernatant was incubated with glutathione-Sepharose for 30 min, washed three times, and resuspended as a 50% slurry in phosphate-buffered saline supplemented with 1% Triton X-100.

Wild-type DOC-2/DAB2 protein and its mutant were expressed in COS cells 24 h after DNA transfection. Cells were collected in 0.5 ml of phosphate-buffered saline supplemented with 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The lysate was sonicated, and then all insoluble material was spun down. The GRB2-GST protein was immobilized on glutathione-Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The supernatant was incubated with glutathione-Sepharose for 30 min, washed three times, and resuspended as a 50% slurry in phosphate-buffered saline supplemented with 1% Triton X-100.

**RESULTS**

**TPA Induces DOC-2/DAB2 Protein Phosphorylation at Serine 24**—DOC-2/DAB2 appears to be a phosphoprotein (8). However, the nature of the phosphorylation and the upstream protein kinase that mediates DOC-2/DAB2 phosphorylation have not yet been elucidated. To study DOC-2/DAB2 protein phosphorylation, NbE cells were treated with the PKC activator TPA (100 ng/ml). Both DOC-2/DAB2 (i.e., p82) and its splicing variant, p59, showed a mobility shift on SDS-polyacrylamide gel electrophoresis (PAGE) within 5 min, and this was sustained for at least 30 min after TPA treatment (Fig. 1A). Transfection of the T7-tagged p82 or p59 expression plasmid into COS or NbE cells (data not shown) followed by TPA treatment also resulted in a similar mobility shift of exogenously expressed DOC-2/DAB2. This finding suggests that a protein modification occurred in DOC-2/DAB2 after TPA treatment.

To determine whether the mobility shift of protein on SDS-PAGE is caused by changes in protein phosphorylation, we labeled T7-p59-transfected cells with [32P]orthophosphate, and cell extracts were immunoprecipitated with the anti-T7 tag antibody 30 min after TPA treatment. A basal level of p59 phosphorylation occurred in control cells, whereas TPA treatment resulted in an increase in p59 phosphorylation (Fig. 1B, left panel). Portions of the immunocomplexes were used to perform Western blot analysis with the anti-T7 tag antibody for equal loading (lower panel). IP, immunoprecipitation; WB, Western blot.
FIG. 2. TPA-induced DOC-2/DAB2 protein phosphorylation at Ser²⁴. A, shown are the predicted sizes of CNBr-digested peptide fragments of ∆B. Three major peptides were generated in the CNBr digestion of ∆B. B–D, COS cells transfected with the indicated T7-tagged DOC-2/DAB2 constructs were metabolically labeled with [³²P]orthophosphate (300 μCi/ml). The T7-tagged immunoprecipitated phosphoproteins were fractionated on a 10% SDS-polyacrylamide gel and visualized by autoradiography (B and C, upper panels). Portions of the immunocomplexes were subjected to Western blot analysis with the anti-T7 tag monoclonal antibody for the control of equal loading (B and C, middle panels). The DOC-2/DAB2 phosphoproteins were excised, and CNBr peptide mapping was performed (B and C, lower panels; and D). WT, wild-type; IP, immunoprecipitation; WB, Western blot; E, ethanol; T, TPA.

has a similar molecular mass compared with ∆B. These results suggest that the N terminus of DOC-2/DAB2 may harbor key phosphoserine residue(s) responsive to TPA.

We have further used ∆B to map the TPA-induced protein phosphorylation site with CNBr phosphopeptide mapping (Fig. 2A). TPA treatment enhanced the phosphorylation of at least two additional peptides, with estimated molecular masses of 13.6/13.2 and 7.3 kDa, respectively, compared with the ethanol control (Fig. 2B). The protein sequence of DOC-2/DAB2 revealed four consensus PKC/CKII phosphorylation sites (i.e., SKKE) at serines 24, 32, 241, and 249. The double mutation of Ser²⁴ and Ser²⁴ (∆B-S24A,S249A) did not affect TPA-induced ∆B phosphorylation (Fig. 2B). However, the double mutation of Ser²⁴ and Ser²⁴ (∆B-S24A,S242A) completely abolished the protein phosphorylation of ∆B by TPA, indicating that phosphorylation of either Ser²⁴ or Ser²⁴ is induced by TPA (Fig. 2B). Therefore, a single mutation of Ser²⁴ or Ser²⁴ was used to precisely map the TPA-induced protein phosphorylation site in DOC-2/DAB2. As shown in Fig. 2C, TPA-induced DOC-2/DAB2 protein phosphorylation was detected only in ∆B and ∆B-S24A, but not ∆B-S24A. In p82-S24A and p59-S24A, the 7.3-kDa phosphopeptide induced by TPA was also eliminated (Fig. 2D). Thus, Ser²⁴ is one of the TPA-induced protein phosphorylation sites in DOC-2/DAB2.

PKC is a DOC-2/DAB2 Protein Kinase—Since S²⁴KK appears to fit the consensus phosphorylation site for either PKC or CKII, we tested whether PKCβII, PKCγ, and CKII could use the synthetic Ser²⁴ peptide (APS₂⁴KKEKKKGSEKTD) of DOC-2/DAB2 as an in vitro substrate. In the presence of PKC activators, all PKC isoforms tested demonstrated comparable PKC activity toward the Ser²⁴ substrate (Fig. 3A). In contrast, CKII failed to phosphorylate the Ser²⁴ peptide, whereas myelin basic protein was phosphorylated significantly (Fig. 3B). The phosphorylation of Ser²⁴ by PKC was abolished when an Ala²⁴ peptide (APA₂⁴KKEKKKGSEKTD) of DOC-2/

FIG. 3. PKC is the DOC-2/DAB2 protein kinase. A, in vitro PKC assay. T7-PKCβII, T7-PKCγ, and T7-PKCb were expressed in COS cells and immunoprecipitated with the anti-T7 tag antibody. The immunocomplex protein kinase assays were performed with the indicated peptides (5 μg/reaction) in the presence or absence of PKC activators. B, in vitro PKC kinase assay using myelin basic protein as a substrate. In the presence of many growth-related genes and increases gene transcription, the PKC activator, p82 elicited a dose-dependent inhibition of TPA-induced AP-1 activity (Fig. 4A). Similarly, expression of p59 and ∆B, but not ∆N, in C4-2 cells
Phosphorylation of DOC-2/DAB2 by Protein Kinase C

Fig. 4. Different effects of DOC-2/DAB2 and its mutants on TPA-induced AP-1 activity. A, dose-dependent inhibition of TPA-induced AP-1 activity by p82; B, deletion of the N terminus of DOC-2/DAB2 abolishes the inhibitory effect of DOC-2/DAB2 on TPA-induced AP-1 activity; C, mutation of Ser\(^{24}\) abolishes the inhibition of TPA-induced AP-1 activity by DOC-2/DAB2. Increasing concentrations of p82 (A) or 0.24 \(\mu\)g of DOC-2/DAB2 expression plasmid (B and C) was cotransfected with \(-73/+63\)-Col-luc and pRSV-\(\beta\)-galactosidase into C4-2 cells. Luciferase activity was determined 24 h after TPA treatment and normalized with \(\beta\)-galactosidase activity. The data represent means \(\pm\) S.D. from three independent experiments.


demonstrated the inhibitory effect on TPA-induced AP-1 activity, indicating that the N terminus of DOC-2/DAB2 is critical in regulating AP-1 activity (Fig. 4B). We also observed a significant decrease in the AP-1 activity of the p59-transfected C4-2 sublines (1) with TPA compared with that of wild-type or control plasmid-transfected C4-2 cells (Table I). Such a decrease suggests that DOC-2/DAB2 protein can function as a potent negative regulator to modulate TPA-induced AP-1 activity.

To further determine the significance of Ser\(^{24}\) phosphorylation of DOC-2/DAB2, we cotransfected a series of mutants with the reporter gene construct into C4-2 cells. As shown in Fig. 4C, expression of \(\Delta B\)-S24A, p82-S24A, and p59-S24A showed greater luciferase activity than p82. Thus, Ser\(^{24}\) in DOC-2/DAB2 is critical for maintaining its inhibitory effect on TPA-induced AP-1 activity. Moreover, \(\Delta B\)-S24A and p82-S24A neutralized the inhibitory activity of \(\Delta B\) and p82 on AP-1 activity in a dose-dependent manner (Fig. 5). In p59-expressing C4-2 cells (Table I), increased expression of p59-S24A also decreased the inhibitory effect of p59 on AP-1 activity. These results clearly indicate that Ser\(^{24}\) phosphorylation of DOC-2/DAB2 plays a critical role in modulating AP-1 inhibitory activity.

N-terminal DOC-2/DAB2 Does Not Interact with GRB2 Protein—Previously, DOC-2/DAB2 was demonstrated to interact with GRB2 (7), which may be a potential underlying mechanism for its AP-1 inhibitory function. Therefore, we tested whether \(\Delta B\) or \(\Delta N\) interacts with GRB2. As expected, in the presence of GRB2, p82, p59, and \(\Delta N\) could be precipitated (Fig. 6, A and B). In contrast, in the presence of ethanol (Fig. 6A) or TPA (Fig. 6B), \(\Delta B\) failed to be precipitated by GRB2 protein. This indicates that the N-terminal DOC-2/DAB2 protein does not interact with GRB2 (Fig. 6, A and B). This interaction appears to be specific because GST-glutathione-Sepharose alone did not precipitate any DOC-2/DAB2 protein (Fig. 6C).

Taken together, these data suggest that the mechanism for the AP-1 inhibitory effect of DOC-2/DAB2 may be mediated by GRB2-independent pathway(s).

DISCUSSION

Although DOC-2/DAB2 is believed to be a candidate tumor suppressor in several cancer cell lines (1–3), the mechanism of its inhibition of cell growth is unknown. In this work, we describe the modulation of DOC-2/DAB2 protein phosphorylation by the PKC activator TPA and the critical role of the phosphorylation in determining its function. We demonstrate that DOC-2/DAB2 serves as a negative regulator in the PKC-mediated signaling axis and may inhibit cell growth through inhibiting AP-1 activity that has been associated with cell proliferation and tumorigenicity (23). In addition to our studies, Xu et al. (7) have shown that the C terminus of DOC-2/DAB2 prevents GRB2 from binding to SOS. Although, the functional impact of this interaction is unclear, it suggests that DOC-2/DAB2 may interfere with the signal transduction pathway activated by receptor protein-tyrosine kinase.

Several mitogenic stimuli, including colony-stimulating factor-1, TPA, phosphatidylinositol-specific phospholipase C, and sphingomyelinase, are known to induce phosphorylation of DOC-2/DAB2. However, unlike the DAB1 protein, which contains a phosphotyrosine moiety, our data and others (8) demonstrate that the major protein phosphorylation site of DOC-2/DAB2 is the serine residue. We mapped a key PKC-modulated DOC-2/DAB2 protein phosphorylation site to Ser\(^{24}\), a conserved residue of DOC-2/DAB2 among human, mouse, and rat species (1). The impact of Ser\(^{24}\) phosphorylation on the function of DOC-2/DAB2 is very significant because mutation of Ser\(^{24}\) to Ala abolishes the inhibitory effect of DOC-2/DAB2 on TPA-induced AP-1 activity (Fig. 4). Interestingly, we observed that the Ser\(^{24}\) mutants of DOC-2/DAB2 can compete with their wild-type proteins in a dose-dependent manner (Fig. 5 and Table I), indicating that Ser\(^{24}\) in DOC-2/DAB2 is a critical amino acid motif modulating its activity. Furthermore, TPA-induced AP-1 activity is substantially inhibited in the p59-expressing C4-2 sublines (i.e. p59-18 and p59-23) (Table I). This may be one of the underlying mechanism(s) contributing to the slower growth of both p59-18 and p59-23 in vitro (1). Similarly, by transfecting the AP-1 reporter construct into NbE cells with detectable endogenous DOC-2/DAB2 levels (1), we found that TPA failed to increase AP-1 activity in this cell line (data not shown). Taken together, these data indicate that DOC-2/DAB2 represents a novel factor in the modulation of the PKC-elicited signaling pathway.

We still do not know how DOC-2/DAB2 inhibits AP-1 activity in the nucleus, although Xu et al. (7) demonstrated that DOC-2/DAB2 can bind to GRB2, which may account for the inhibition of the GRB2-mediated signaling pathway. However, our results indicate that N-terminal DOC-2/DAB2 (i.e. \(\Delta B\)), with or without phosphorylation, does not interact with GRB2 (Fig. 6). Therefore, this AP-1 inhibitory effect must be mediated by GRB2-independent pathway(s). Alternatively, DOC-2/DAB2 may inhibit the function of signal molecules in PKC-elicited pathway(s). However, we observed that Ser\(^{24}\) and Ser\(^{24}\)/Ser\(^{22}\) mutants appear to affect the phosphorylation status of other serine residues as indicated by CNBr phosphopeptide mapping (Fig. 2), suggesting a critical role for Ser\(^{24}\) phosphorylation in modulating the protein conformation of DOC-2/
Moreover, analysis of the amino acid sequence surrounding Ser24 revealed a potential nuclear localization signal (S24KKEKKKG). The sequence KKEK has been shown to associate with the actin-interacting capability of villin (23), suggesting that the subcellular localization of DOC-2/DAB2 may be affected by protein phosphorylation. Interestingly, we observed that TPA induces accumulation of DOC-2/DAB2 in the particulate fraction (i.e. membrane fraction) of prostatic epithelial cells. 2 The accumulated DOC-2/DAB2 protein in the particulate fraction appears to be phosphorylated as determined by the mobility shift on SDS-PAGE. This observation is in accord with reports suggesting that phosphorylation within the sequence immediately N-terminal to the minimal nuclear localization signal modulates the transport kinetics of the respective protein (24, 25). The effect of Ser24 phosphorylation on DOC-2/DAB2 protein localization, which may explain how DOC-2/DAB2 regulates AP-1 activity, warrants further investigation.

Another important issue is the mechanism controlling the phosphorylation of Ser24. Among the protein kinases we tested (PKC\(\beta\)II, PKC\(\gamma\), PKC\(\delta\), and CKII), PKC has been implicated in many aspects of cellular functions, including cell proliferation and differentiation, T-cell activation, and gene activation (10–15). Although functional redundancies between PKC isoforms have been shown, other reports suggest that individual PKCs may have distinct functions (10–15, 26–

**TABLE I**

| Cell lines | Exp. 1 plasmid control\(^a\) |
|------------|-------------------------------|
| Wild-type C4–2 | 96 ± 10\(^c\) |
| Neo-4 | 84 ± 15 |
| p59–18 | 25 ± 5 |
| p59–23 | 16 ± 4 |
| Exp. 2 plasmid control\(^b\) | 120 ± 20 |
| Exp. 3 Plasmid control\(^b\) | 106 ± 12 |
| p59-S24A \(^a\) | 98 ± 14 |

\(^a\) The plasmid control (pCI-neo; 0.24 \(\mu\)g) was used in these experiments.

\(^b\) The plasmid control (pCI-neo; 2.4 \(\mu\)g) and p59-S24A (0.24 \(\mu\)g) were used in this experiment.

\(^c\) The data were calculated from three independent transfections; the numbers represent the mean ± S.D.

**FIG. 5.** Mutation of Ser\(^{24}\) abolishes the inhibitory effect of DOC-2/DAB2 on TPA-induced AP-1 activity. A, dose-dependent effect of \(\Delta B\)-S24A on antagonizing the inhibitory activity of \(\Delta B\) on TPA-induced AP-1 activity; B, dose-dependent effect of p82-S24A on antagonizing the inhibitory activity of p82 on TPA-induced AP-1 activity. Increasing concentrations of \(\Delta B\)-S24A (A) or p82-S24A (B) were cotransfected with \(-73/-63\)-Col-luc and pRSV-\(\beta\)-galactosidase into C4-2 cells. Luciferase activity was determined 24 h after TPA treatment and normalized with \(\beta\)-galactosidase activity. The data represent means ± S.D. from three independent experiments.

**FIG. 6.** Interaction of GRB2 and DOC-2/DAB2 protein. COS cells (8 \(\times\) 10\(^5\) cells/60-mm Petri dish) were transfected with each indicated cDNA construct (4 \(\mu\)g) for 24 h. After incubation with either ethanol (A) or TPA (B and C) for 30 min, cell lysates were prepared from each condition. An aliquot of recombinant GRB2-GST-glutathione-Sepharose (\(-200 \mu\)g; A and B) or GST-glutathione-Sepharose (C) was incubated with 400 \(\mu\)l of cell lysates. After overnight incubation and washing, bound proteins were subjected to SDS-PAGE analyses and blotted with either the anti-T7 tag antibody for DOC-2/GRB2 (upper panels) or the anti-GST antibody for GRB2 (middle panels). An aliquot of each cell lysate (\(-30 \mu\)g) was also subjected to SDS-PAGE analysis and blotted with the anti-T7 tag antibody (lower panels) as an internal control. IP, immunoprecipitation; WB, Western blot.
28) and substrate specificity (13, 29–30). Similarly, as shown in Fig. 3E, we observed a slightly different phosphorylation profile for PKCδ. The largest phosphopeptide is not phosphorylated by PKCδ. This suggests the distinct activity of PKC isoforms toward the phosphorylation of ΔB. In addition, there was an increased expression of both DOC-2/DAB2 and PKCδ mRNAs in the enriched basal epithelial cells of degenerated prostate in castrated rat (1), suggesting the involvement of PKCδ in the control of DOC-2/DAB2 activity in prostate gland homeostasis. Kinetic studies with purified recombinant PKC will be required to further elucidate whether Ser24 of DOC-2/DAB2 is a preferential substrate for any PKC isoform.

In summary, our results indicate that DOC-2/DAB2 is phosphorylated at Ser24 by PKC, which plays a critical role in controlling AP-1 activity. The regulation of DOC-2/DAB2 protein phosphorylation may in turn regulate its inhibitory function in cell proliferation and tumorigenicity. It may define a novel mechanism for the transduction of negative growth signals from the membrane to the nucleus.

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