The Inhibitory Role of DOC-2/DAB2 in Growth Factor Receptor-mediated Signal Cascade

DOC-2/DAB2-MEDIATED INHIBITION OF ERK PHOSPHORYLATION VIA BINDING TO Grb2

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DOC-2/DAB2 (differentially expressed in ovarian carcinoma-2/disabled 2) appears to be a potential tumor suppressor gene with a growth inhibitory effect on several cancer types. Previously, we have shown that DOC-2/DAB2 suppresses protein kinase C-induced AP-1 activation, which is modulated by serine 24 phosphorylation in the N terminus of DOC-2/DAB2. However, the functional impact of the C terminus of DOC-2/DAB2, containing three proline-rich domains, has not been explored. In this study, we examined this functional role in modulating signaling mediated by peptide growth factor receptor tyrosine kinase, particularly because it involves the interaction with Grb2. Using sequence-specific peptides, we found that the second proline-rich domain of DOC-2/DAB2 is the key binding site to Grb2 in the presence of growth factors. Such elevated binding interrupts the binding between SOS and Grb2, which consequently suppresses downstream ERK phosphorylation. Reduced ERK phosphorylation was restored when the binding between DOC-2/DAB2 and Grb2 was interrupted by a specific peptide or by increasing the expression of Grb2. Furthermore, the C terminus of the DOC-2/DAB2 construct can inhibit the AP-1 activity elicited by growth factors. We conclude that DOC-2/DAB2, a potent negative regulator, can suppress ERK activation by interrupting the binding between Grb2 and SOS that is elicited by peptide growth factors. This study further illustrates that DOC-2/DAB2 has multiple effects on the RAS-mediated signal cascades active in cancer cells.

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† The abbreviations used: SH, Src homology; RPTK, receptor protein tyrosine kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; NT3, neurotrophin 3; ERK, extracellular signal-related protein kinase; MAP, mitogen-activated protein; Grb2, growth receptor binding protein 2.

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EXPERIMENTAL PROCEDURES

Cell Lines, Synthetic Peptides, and Plasmid Constructs—A rat pheochromocytoma cell line (PC12) was grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated horse serum (Life Technologies, Inc.), 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. C4-2, NbE, and COS cells were maintained in T medium supplemented with 5% fetal bovine serum (3). Four peptides were synthesized corresponding to the protein sequence of DOC-2/DAB2: PPQ (amino acids 619–627), PPK (amino acids 714–722), PPL (amino acids 663–671), and LLL (amino acids 663–671), the substitution of proline with leucine). All DOC-2/DAB2 cDNA expression constructs, ...
Western blot analysis detected by the antibody against T7 tag (twice with lysis buffer, dissolved in sample buffer, and subjected to PLUS-agarose (Santa Cruz Biotechnology, Inc.). The pellet was washed after a low speed spin, 20 μl of supernatant was separately incubated with anti-DOC-2/DB2 antibody, αp96 (A and B) or the anti-T7 tag antibody, αT7 (C and D). The lower panels, an aliquot of cell lysate was analyzed using Western blot detected by either αp96 (A and B) or αT7 (C and D). IB, immunoblot.

such as pCI-neo-T7-p82 (T7-p82) and pCI-neo-T7-ΔN (T7-ΔN), have been described (7).

Transfection of Plasmid Vector and Oligopeptide—The indicated number of cells was plated at 37 °C 24 h prior to LipofectAMINE transfection (Life Technologies, Inc.). In each experiment, the control plasmid (pCI-neo) was supplemented to reach an equal amount of total DNA. For the NT3 induction experiment, the trkC expression vector (10), pAC-CMV-trkC, was co-transfected with the DOC-2/DB2 cDNA construct. After transfection, cells were switched to a low serum condition (1% heat-inactivated horse serum for PC12 cells; 0.5% fetal bovine serum for COS and C4-2 cells) for another 24 h prior to being treated with growth factors. For peptide transfection, cells were plated in a 24-well plate with serum-free medium for 24 h. Chiator™ reagent (Active Motif) was mixed with 10 ng of different oligopeptides according to the manufacturer's protocol. 1 h after transfection, cells were treated with growth factors, and cell lysate was prepared at the indicated time.

In Vitro GST-Grb2 Binding Assay—After transfection, cells were exposed to 50 ng/ml of EGF or recombinant NT3 (Upstate Biotechnology). The cells were collected in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA supplemented with 1% of Triton X-100, and a mixture of protease inhibitors) at the indicated time. After a low speed spin, 0.4 ml of supernatant was separated and incubated overnight at 4 °C with either 60 μl of GST-Grb2-glutathione Sepharose or GST-glutathione Sepharose. After centrifugation, the pellet was washed twice with the lysis buffer, dissolved in the sample buffer, and then subjected to Western blot analysis probed with either monoclonal antibody against DOC-2/DB2 (αp96) (Transduction Laboratories) or against the T7 tag (αT7) (Novagen).

Co-immunoprecipitation Assay—The cells were co-transfected with both DOC-2/DB2 expression vectors and 0.6 μg of Grb2 expression vector (pHM6-Grb2). Using the same treatment protocol, cells were collected in 0.5 ml of lysis buffer. After a low speed spin, 0.4 ml of supernatant was incubated overnight at 4 °C with 1 μl of monoclonal antibody against Grb2 (Transduction Laboratories) and 40 μl of protein G PLUS-agarose (Santa Cruz Biotechnology, Inc.). The pellet was washed twice with lysis buffer, dissolved in sample buffer, and subjected to Western blot analysis detected by the antibody against T7 tag (αT7).

ERK Phosphorylation Assay—Transfected cells were exposed to 50 ng/ml of EGF or NT3 for 10 min and were collected in 70 μl of phosphate-buffered saline (with 1% of Triton X-100 and a mixture of protease inhibitors). After a low speed spin, 20 μl of supernatant was subjected to Western blot analysis. The filter was probed with the antibody against phosphorylated ERK p44/42 (New England Biolab), and the same filter was stripped and reprobed with antibodies against either total ERK 1/2 (p44/42) or p42 (New England Biolab).

Luciferase Reporter Gene Assay—The AP-1-Luciferase reporter construct (-73/-63-Col-Luc) (7) and internal control β-gal vector (pCH110) were used with various DOC-2/DB2 cDNA expression vectors. The cells were treated with or without 50 ng/ml of EGF or recombinant NT3 for 16 h. Both luciferase and β-galactosidase were assayed (7). The data from the reporter gene activity were normalized with β-galactosidase activity.

RESULTS

NT3 and EGF Increase the Binding of Grb2 to DOC-2/DB2—In a previous study (7), we showed that the C terminus, but not the N terminus, of DOC-2/DB2 binds to Grb2. In this study, we investigated the effect of growth factors on this binding. Because DOC-2/DB2 is expressed in brain tissue (4) and prostate epithelia (3), we chose two RPTK systems: NT3/trkC cascade in PC12 cell and EGF/EGFR cascade in C4-2 cells. PC12 cells were co-transfected with pAC-CMV-trkC and T7-p82 cDNAs and then treated with NT3 (50 ng/ml) for the indicated time. The cell lysate was prepared and subjected to Grb2-GST binding assay. As shown in the upper panel of Fig. 1A, a basal level interaction between DOC-2/DB2 and Grb2 was detected in the PC12 cells; a similar result was shown previously (7). However, binding of DOC-2/DB2 to Grb2 increased 5 min after treatment with NT3, and increased binding remained constant throughout the entire course of treatment. This indicates that trkC activation can immediately increase the interaction between DOC-2/DB2 and Grb2. The same expression levels of DOC-2/DB2 expression were detected in each lane (Fig. 1A, lower panel), and this rules out the possibility of a transfection artifact.

In the other RPTK signaling system (Fig. 1B, upper panel),
increased binding of DOC-2/DAB2 to Grb2 occurred in a time-
dependent manner in a prostatic epithelial cell line (C4-2)
treated by EGF. Also, as shown in the lower panel of Fig. 1B,
an equal amount of DOC-2/DAB2 protein expression was detected
at each time point. There was difference in the Grb2 binding
kinetics between NT3-treated PC12 cells and EGF-treated
C4-2 cells. For example, EGF showed a slower rate of Grb2
binding than did NT3. Peak binding of Grb2 occurred 20 min
after EGF treatment, compared with 5 min after NT3 treat-
ment. Such difference may influence the effect of DOC-2/DAB2
on the downstream pathway mediated by these two growth
factors.

In addition to data obtained from the in vitro binding assay,
we used co-immunoprecipitation to examine the intracellular
interaction between DOC-2/DAB2 and Grb2. As shown in Fig.
1 (C and D), the complex containing DOC-2/DAB2 significantly
increased in the presence of either NT3 or EGF compared with
the control. EGF also increased the intracellular interaction
between the Grb2 and DOC-2/DAB2. Data from both Grb2 and
co-immunoprecipitation assays indicate that the interaction
between DOC-2/DAB2 and Grb2 was enhanced under the stim-
ulation of peptide growth factors, which suggests that DOC-2/
DAB2 may play a regulatory role in RPTK-mediated signaling.

Growth Factors Enhance the Binding of the C Terminus of
DOC-2/DAB2 to Grb2—As we described, the C terminus but
not the N terminus of DOC-2/DAB2 interacts with Grb2 (7).
To determine whether growth factors increase the affinity of the C
terminus of DOC-2/DAB2 to Grb2, we performed Grb2 binding
assay using the N-terminal deletion mutant of DOC-2/DAB2
(T7-ΔN). We wanted to validate this interaction using different
cell types. Therefore, COS cells were reconstituted with trkC in
the presence of either T7-p82 or T7-ΔN construct. As expected,
stimulation with NT3 significantly increased the binding of
Grb2 to both DOC-2/DAB2 and the C terminus of DOC-2/DAB2
(Fig. 2A). This elevated binding was not caused by variable
expressions of T7-p82 or T7-ΔN proteins because equal
amounts of these proteins were detected. Similarly, Grb2 bind-
ing to DOC-2/DAB2 or to the C terminus of DOC-2/DAB2 also
increased with EGF treatment (Fig. 2B). These data indicate
that the binding domain of DOC-2/DAB2 to Grb2 is located in
the C terminus of DOC-2/DAB2.

Binding of Grb2 Is Mediated by the Second Proline-rich
Domain of DOC-2/DAB2—There are three proline-rich domains
in DOC-2/DAB2 with the same consensus sequence as the SOS

FIG. 3. The specific interaction of the proline-rich domains
in DOC-2/DAB2 and Grb2. A, COS cells (6 × 10⁵/100-mm dish) were
transfected with either DOC-2/DAB2 (T7-p82) or the C terminus of
DOC-2/DAB2 (T7-ΔN). The cell lysates were prepared 48 h after trans-
faction and subjected to Grb2 binding assay in the presence of different
concentrations of oligopeptides. B, cell lysate from 80% confluent NbE
cells were prepared and subjected to Grb2 binding assay in presence of
different concentrations of oligopeptides. The precipitate was detected
by either anti-T7 (αT7) or anti-p96 (αp96) antibodies.

FIG. 4. The interruption of SOS binding to Grb2 by DOC-2/
DAB2. COS cells (6 × 10⁵/60-mm dish) were co-transfected with HA-
Grb2 and either pCI-neo or DOC-2/DAB2 (T7-p82) and then treated
with EGF (100 ng/ml) for 10 min. The cell lysates were prepared
subjected to co-immunoprecipitation assay by anti-HA tag antibody
(αHA). The precipitate was detected by anti-SOS antibody (αSOS) (top
panel) and reprobed with anti-Grb2 antibody (αGrb2) (middle panel).
Total cell lysate (bottom panel) was detected by anti-p96 antibody
(αp96). IB, immunoblot.

FIG. 5. The suppression of the growth factor-induced ERK phos-
phorylation mediated by sequestering Grb2 by DOC-2/DAB2 protein.
PC12 (A and C) and C4-2 (B and D) cells (8 ×
10⁵/10-cm² plate) were co-transfected with either the control plasmid pCI, full-
length (T7-p82), or the C terminus of
DOC-2/DAB2 (T7-ΔN) and pAC-CMV-
trkC (A and C) or EGFR expression plas-
mid (B and D). In addition, pHM-Grb2 (C,
D) was used for increasing Grb2 levels in
cells, 24 h after transfection, cells were
switched to a low serum medium for an-
other 24 h: 50 ng/ml of NT3 (A and C) or
EGF (B and D) were added for a 10-min
incubation. An aliquot of cell lysate was
analyzed by Western blot. In the upper
panels, the filter was probed with an anti-
body against phosphorylated ERK
p44/42 (αERK) and, in the lower panels,
the same filter was reprobed with either
ERK p42-specific antibody (αERK2) or
ERK1/2-specific antibody (αERK1/2). IB,
immunoblot.
binding site to Grb2. Therefore, all three domains may be involved in Grb2 binding. To compare the binding affinity of these proline-rich sequences, four oligopeptides were synthesized according to the DOC-2/DAB2 protein sequence: PPQ (amino acids 619–627); PPL (amino acids 663–669); LLL (amino acids 663–669 with all proline residues substituted with leucine residues); and PPK (amino acids 714–722). Using the in vitro GST-Grb2 binding assay, the PPL peptide blocked the binding of either T7-p82 or T7-ΔN proteins to GST-Grb2, whereas the PPQ peptide had no effect (Fig. 3A). Moreover, we prepared cell lysates from NbE cells (a normal rat prostatic epithelial cell line with the endogenous expression of DOC-2/DAB2 proteins) to determine the in vitro binding of DOC-2/DAB2 proteins to Grb2. As shown in Fig. 3B, PPL effectively blocked the binding in a dose-dependent manner compared with the other two peptides (i.e. PPQ and PPK). The effective dose of PPL in COS and NbE cells was very similar, suggesting that the binding kinetics of these proteins remains consistent in different cell lines. The proline residues in this domain are critical for binding because the peptide with leucine substitution (i.e. LLL) cannot affect the binding between DOC-2/DAB2 and Grb2 (Fig. 3B). These data clearly indicate that the second proline-rich domain in the C terminus of DOC-2/DAB2 is the key interactive site with Grb2.

DOC-2/DAB2 interrupts the binding of Grb2 with SOS Interaction between the Grb2 protein, and the activated receptor can initiate translocation of a group of proteins, namely guanine nucleotide exchange factors (such as SOS), which modulate the GTPase activity of RAS. As shown in Fig. 4, stimulation with EGF increased the binding of Grb2 to SOS in COS cells with no detectable levels of DOC-2/DAB2. However, increased expression of p82 levels in COS cells diminished the binding of Grb2 to SOS, which indicates that DOC-2/DAB2 and SOS compete for the same SH3 site in Grb2.

Both Native DOC-2/DAB2 and the C Terminus of DOC-2/DAB2 Inhibit NT3- and EGF-induced ERK Activation—Grb2 is a key adapter protein for the growth factor-induced MAP kinase pathway (11). To understand the impact of the interaction between DOC-2/DAB2 and Grb2 on this pathway, we examined the phosphorylation status of ERK kinase in the presence of DOC-2/DAB2. Upon the treatment of growth factors, ERK phosphorylation reflected the activation of ERK kinase (12). ERK activation is transient. It peaks at 10 min and then returns to a basal level within 30 min. As shown in Fig. 5A, the basal level of phosphorylated ERK was very low in PC12 cells without NT3 treatment. The addition of DOC-2/DAB2 did not alter this level.

In contrast, increasing phosphorylation of ERK1 (44 kDa) and ERK2 (42 kDa) was detected in PC12 cells 10 min after NT3 treatment. Furthermore, NT3-induced phosphorylation of ERK1/2 decreased profoundly in the presence of either full-length DOC-2/DAB2 (T7-p82) or the C terminus of DOC-2/DAB2 (T7-ΔN). Such a change was not caused by the decrease of ERK1/2 protein levels (Fig. 5A, middle and lower panel).

Without EGF treatment, the basal level of ERK2 phosphorylation was lower in C4-2 cells than in PC12 cells (Fig. 5B). With EGF treatment, phosphorylation levels of ERK kinases, mainly ERK2 (42 kDa), were elevated. The presence of either T7-p82 or T7-ΔN dramatically suppressed EGF-induced ERK2 phosphorylation. However, the steady-state levels of the ERK2 protein remained the same. These data indicate that ERK2 phosphorylation was inhibited when DOC-2/DAB2 proteins decrease the availability of Grb2 to SOS.

Decreased ERK phosphorylation appears to be a potent downstream event that results from the binding between DOC-2/DAB2 and Grb2. We examined whether increased expression of Grb2 restores the ERK phosphorylation status in cells treated with growth factors. As shown in Fig. 5 (C and D), in the absence of either NT3 or EGF, transfection of the Grb2 expression vector failed to induce ERK phosphorylation because Grb2 alone is not capable of activating the growth factor-induced downstream signal pathway (8). In contrast, in either EGF-treated C4-2 or NT3-treated PC12 cells, transfection of the Grb2 expression vector did restore the decreased ERK phosphorylation induced by T7-p82 proteins. This suggests that DOC-2/DAB2 binding to Grb2 is a key step in inactivating ERK phosphorylation.
Because the second proline-rich domain of DOC-2/DAB2 is a key binding site to Grb2, we investigated whether blocking this binding also affects the ERK phosphorylation status in cells stimulated with growth factors. To do this, NbE cells were transfected with either PPL or LLL peptide, and the ERK phosphorylation status in EGF-treated NbE cells was determined. As shown in Fig. 6 (A and B), minutes after EGF treatment, the ERK2 phosphorylation increased about 3–4-fold compared with basal level. PPL enhanced ERK2 phosphorylation about 20-fold. It peaked 10 min after EGF treatment, whereas LLL failed to have the same effect, indicating that PPL can specifically prevent the sequestering Grb2 by the second proline-rich domain of DOC-2/DAB2. These “free” Grb2 molecules amplify signal transduction via the downstream cascade, such as the activation of ERK kinase. Similarly, PPL but not LLL can also antagonize the inhibitory effect of DOC-2/DAB2 on EGF-induced gene transcription (Fig. 6 C).

The overall activity of the reporter gene in this experiment was lower than usual because cell death became more apparent when cells were incubated with both LipofectAMINE and Chariot™ transfection agents. Nevertheless, these data further indicate that one mechanism of DOC-2/DAB2 is to balance the growth factor-induced signal by modulating the availability of effector protein such as Grb2.

**DISCUSSION**

Grb2 is a potent adaptor protein. It contains both SH2 and SH3 domains in modulating RPTK-elicited signaling. The SH2 domain directly recognizes phosphotyrosine motifs and is thereby recruited to activated, phosphorylated RPTK. This interaction helps the recruitment of guanine nucleotide exchange factors (i.e. SOS) through the binding of the SH3 domain, which subsequently stimulates RAS-GTP binding. The RAS-mediated MAP kinase cascade, a key pathway controlling growth and differentiation, further leads to gene transcription by activating several transcription factors. We and others have shown that DOC-2/DAB2 can interact with Grb2 through its C terminus (7, 15). However, the functional role of this interaction has not been elucidated.

In this study, we manipulated PC12 cell lines without DOC-
2/DAB2 expression such as C4-2 and with different DOC-2/DAB2 cDNA constructs. We found that activation of RPTK by its own ligand also increases the binding of DOC-2/DAB2 to Grb2, which interrupts the binding of SOS to Grb2 (Figs. 1 and 4). We also demonstrated that the second proline-rich domain in the C terminus of DOC-2/DAB2 protein is the key site responsible for the Grb2 binding that prevents a sequential change in the downstream region of the RPTK-mediated pathway (Figs. 3 and 5), including suppressing ERK phosphorylation.

We used a cell line (NbE) that expresses endogenous DOC-2/DAB2 to demonstrate the interaction between endogenous DOC-2/DAB2 and Grb2 proteins. We showed that this interaction appears to be sequence-specific because only peptides with the same sequence as the second proline-rich domain of DOC-2/DAB2 can interrupt Grb2 binding, whereas substitution of key proline residues with leucine residues abolishes its inhibitory function (Fig. 3). Moreover, increasing Grb2 expression in C4-2 cells by using the transient transfection of the Grb2 expression vector alters the inhibitory effect of DOC-2/DAB2 (Fig. 5). We conclude that this inhibitory effect on the MAP kinase pathway results from sequestering Grb2 away from recruiting downstream signaling effectors such as SOS. Therefore, DOC-2/DAB2 represents a potent homeostatic factor that modulates many exogenous-stimulus-mediated signal pathways.

To characterize the effect of DOC-2/DAB2 on the RPTK-mediated pathway, we studied two classic ligands in three different cell lines, NT3, which interacts with its receptor trkC, is an important neurotrophin for neuronal development and plasticity. Because other members of the disabled family (i.e., DAB1) have been associated with neuronal development (16, 17) and DOC-2/DAB2 is present in brain tissue, we employed PC12 cells (a well established cell model for studying neuronal differentiation) to examine the possible role of DOC-2/DAB2 in NT3-induced signaling. Our results (Figs. 1A and 3A) demonstrate that DOC-2/DAB2 can block the transient activation of ERK kinase that is considered a mitogenic signal (18) even though neurotrophins can induce a sustained activation of ERK kinase, which can last for several hours in PC12 cells and is related to neuronal differentiation (19). However, we failed to observe any inhibition of the second phase of ERK activation in the presence of DOC-2/DAB2 (data not shown). These results imply that DOC-2/DAB2 may modulate the mitogenic effect of NT3. This function differs from DAB1 protein in neuronal cells. EGF, a potent mitogen for cell growth, elevation, and EGFR amplification and/or mutation is associated with the progression of many cancer types (20) including prostate cancer (21).

Data from our laboratory and others indicate that DOC-2/DAB2 is a tumor suppressor, which is absent in many cancer types (1–6). In this study, we demonstrated that the C terminus of DOC-2/DAB2 may block EGFR-mediated signaling by sequestering Grb2 (Fig. 1) from the upstream of the cascade. This blocking essentially deactivates several key effectors such as ERK and AP-1 (Figs. 3 and 7). Several studies show that unregulated activation of ERK can cause cell transformation (22) and that AP-1 activation is crucial to angiogenesis and neoplastic invasion (23). Moreover, our recent publication indicates that the serine 24 phosphorylation of DOC-2/DAB2 inhibits protein kinase C-mediated gene activation (7). Taken together, we believe that DOC-2/DAB2 represents a unique negative regulator. Upon exogenous stimulus, it demonstrates multiple actions on signaling cascade.

The underlying mechanism regarding the increased affinity of DOC-2/DAB2 to Grb2 by growth factors is still unknown. Our data (Figs. 1 and 2) indicate that this is a rapid event and that the steady-state levels of DOC-2/DAB2 do not change. Therefore, post-translational modification, such as phosphorylation, is likely. Unlike with DAB1 (16), we did not detect any tyrosine phosphorylation in DOC-2/DAB2 (data not shown). Xie et al. (24) report that colony-stimulating factor-1 induces serine phosphorylation in DOC-2/DAB2, but they did not determine the key amino acid(s) responsible for this event. We demonstrated that the phorbol ester also induces serine phosphorylation in the N terminus but not in the C terminus of DOC-2/DAB2 (7). Therefore, post-translational modification of DOC-2/DAB2 may play a role in this event because there are several potential ERK phosphorylation sites in the C terminus of DOC2/DAB2 (24). Alternatively, however, we cannot rule out that the presence of other factor(s) may facilitate this interaction upon stimulation with growth factors. Therefore, more detailed study is warranted.

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