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

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Received for publication, October 17, 2002, and in revised form, November 27, 2002
Published, JBC Papers in Press, December 8, 2002, DOI 10.1074/jbc.M210628200

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

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

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

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

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

EXPERIMENTAL PROCEDURES

Cell Lines, Synthetic Peptides, and Plasmid Constructs—LNCaP, NBE, and COS cells were maintained in T medium supplemented with 5% fetal bovine serum (3). The following peptides were synthesized according to the amino acid sequence of DOC-2/DAB2: PPQ (amino acid 619–627); LLQ (amino acid 619–627, proline to leucine); PPL (amino acid 663–671); LLL (amino acid 663–671, proline to leucine); and PPK (amino acid 714–722) (6). All DOC-2/DAB2 cDNA constructs, pC1-neo-T7-p82 (p82) and pC1-neo-T7-ΔN (ΔN), and GST-Grb2 have been described previously (6).

Cell Transfection—For transient transfection, cells were plated 24 h prior to transfection using LipofectAMINE PLUS reagent (Invitrogen). In each experiment, the control plasmid (pC1-neo) was supplemented to make an equal amount of total DNA. Twenty-four h after transfection, cells were switched to serum-free T medium for another 24 h prior to the treatment with 50 ng/ml EGF (Upstate Biotechnology).

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

GST Pull-down and Co-immunoprecipitation Assay—For GST pull-down assay, cells were exposed to 50 ng/ml of EGF, and cell lysate was collected in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA supplemented with 1% Triton X-100, and a mixture of protease inhibitors) at the indicated time. After a low speed spin, 0.4 ml of supernatant was separately incubated with either 30 µl of GST-glutathione-Sepharose or GST fusion protein-Sepharose overnight at 4 °C. The next day, the pellet was washed twice with lysis buffer and dissolved in the sample buffer and then subjected to Western blot analysis probed with antibodies against DOC-2/DAB2 (α96) (Transduction Laboratories) or against T7 tag (αT7) (Novagen).
For co-immunoprecipitation, cell lysate was collected in 0.5 ml of lysis buffer. After a low speed spin, 0.4 ml of supernatant was incubated with 1/9 H9262 g of antibody against Src (Oncogene Research Products) and then 40/9 H9262 l of protein A-Sepharose (Amersham Biosciences) overnight at 4 °C. The pellet was washed twice with lysis buffer and dissolved in sample buffer and then subjected to Western blot analysis detected by T7.

Detection of c-Src and Erk2 Protein Phosphorylation—For determining phosphorylation status of Src, cells were transfected with Src expression vectors and exposed to 50 ng/ml EGF for 10 min. Cell lysate was collected in 70/9 H9262 l of phosphate-buffered saline (with 1% Triton X-100 and a mixture of protease inhibitors). After a low speed spin, 20/9 H9262 l of supernatant was subjected to Western blot analysis. The filter was probed with the antibody against phosphorylated Src antibodies against either tyrosine 416 (pSrc416) (Upstate Biotechnology) or tyrosine 527 (pSrc527) (Cell Signaling), and the same filter was stripped and reprobed with the antibody against total Src (Src) (Oncogene).

For determining phosphorylation status of Erk2 protein, cells were transfected with hemagglutinin-Erk2 and exposed to 50 ng/ml EGF for 10 min. Cell lysate was collected in 0.5 ml of lysis buffer. After a low speed spin, 0.4 ml of supernatant was immunoprecipitated with hemagglutinin-Matrix (Covance). After washing twice with lysis buffer, the pellets were added with sample buffer and subjected to Western blot analysis. The filter was probed with the antibody against phosphorylated extracellular signal-regulated kinase p44/42 (pErk) (Cell Signaling), and the same filter was stripped and reprobed with antibody against either total extracellular signal-regulated kinase 1/2 (Erk) or p42 (Erk2) (Cell Signaling).

**RESULTS**

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

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**FIG. 1.** Differential interaction between the proline-rich domain of DOC-2/DAB2 and several proteins containing SH3 domain. A, COS cells (4 × 10^6/60-mm dish) were co-transfected with either p82 (top panel) or AN (bottom panel) for 48 h. An equal amount of cell lysate was incubated with the indicated GST/SH3 domain fusion proteins. A pull-down assay was performed, and the Western blot was analyzed with αT7. B, cell lysate prepared from NbE cells was subjected to a pull-down assay using GST-SH3 (Src) (top panel) or GST-SH3 (Grb2) (bottom panel) with increasing amounts of peptides. The amount of DOC-2/DAB2 from each lane was determined by Western analysis using αp96. The number underneath each lane represented the relative level of DOC-2/DAB2 in each treatment compared with that in control (1). C, a schematic representation of the SH3 domain in DOC-2/DAB2 interacting with either Grb2 or c-Src.

**FIG. 2.** Increased association of DOC-2/DAB2 with c-Src in cells treated with EGF. A, COS cells (6 × 10^6/100-mm dish) were co-transfected with c-Src and p82 vectors. Twenty min after EGF treatment, cell lysate was subjected to a co-immunoprecipitation assay. B, LNCaP cells (8 × 10^6/100-mm dish) were treated with EGF, and cell lysate was collected at the indicated time. The pull-down was performed using GST-Src and then Western blot was performed using αp96 (top panel). Equal amounts of cell lysate from each sample were analyzed by Western blot using αp96 (bottom panel). C, the profile of the DOC-2/DAB2/c-Src complex in LNCaP cells after EGF treatment. Data (mean ± S.E.) were calculated from experiments performed in duplicate.
domains indicating that the potential interaction with other proteins containing SH3 domain, in addition to Grb2 (4–6), should be expected. To screen the interaction with proteins containing SH3 domain, we performed pull-down experiment using several GST fusion proteins derived from three groups of proteins containing SH3 domain with different functions. For adapter proteins, Grb2 has been shown to interact with p82 (non-spliced DOC-2/DAB2 protein) previously (4–6); Crk did not show any detectable interaction with p82 (Fig. 1A). For the non-receptor Src tyrosine kinase family, both c-Src and Fgr could interact with p82 (Fig. 1A). However, no detectable interaction was shown between p82 and the SH3 domain of spectrin, an actin-binding protein (Fig. 1A). Apparently, this interaction required the C-terminal of DOC-2/DAB2, because the N-terminal deletion of DOC-2/DAB2 (ΔN) (5) had much stronger interaction compared with p82 (Fig. 1A, lower panel).

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

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

EGF Enhances the Association of DOC-2/DAB2 with c-Src—To further verify the interaction of Src with DOC-2/DAB2 intracellularly, we performed co-immunoprecipitation assays. As shown in Fig. 2A, when both c-Src and DOC-2/DAB2 were co-expressed in COS cells, the DOC-2/DAB2 was detected in the immunocomplex precipitated with aSrc. The amount of DOC-2/DAB2 in the immunocomplex was substantially increased when the cells were treated with EGF. Similarly, in LNCaP cells, the association of c-Src with DOC-2/DAB2 is promptly elevated in a time-dependent manner after EGF treatment (Fig. 2B). The interaction peaked at 20 min after EGF treatment and remained unchanged for 60 min, indicating that c-Src had a prolonged interaction with DOC-2/DAB2.

We further determined whether EGF was able to increase the affinity between c-Src and DOC-2/DAB2. We measured the dissociation constant between c-Src and DOC-2/DAB2 in the presence of PPQ. As shown in Fig. 3, the increasing amount of PPQ caused the dissociation between c-Src and DOC-2/DAB2. However, in the presence of EGF, the IC50 of PPQ was 259 μM compared with the IC50 of PPQ (168 μM) in the absence of EGF. Taken together, these data indicated that EGF could facilitate the interaction between c-Src and
DOC-2/DAB2 because of the increased the affinity between both proteins.

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

To avoid any transfection artifact, we decided to take another approach by interrupting the interaction between DOC-2/DAB2 and c-Src in NbE cells expressing endogenous DOC-2/DAB2 and c-Src proteins using synthetic peptides. PPQ that inhibits the interaction between DOC-2/DAB2 and c-Src (Fig. 2B) was transfected into NbE cells. LLQ with the substitution of proline with leucine was used as a negative control. As shown in Fig. 5A, the phosphorylation of c-Src was determined by using opSrc^{416} in NbE cells after EGF treatment. The intensity of Tyr-416 phosphorylation was measured and normalized with the total Src (Fig. 5B). In the absence of blocking peptide, EGF induced Tyr-416 phosphorylation (~3-fold). Apparently, PPQ could antagonize the interaction between DOC-2/DAB2 and c-Src and significantly increased the Tyr-416 phosphorylation (~4- to 6-fold) in NbE cells without or with EGF treatment. Although LLQ slightly increased Tyr-416 phosphorylation (~2-fold), maybe because of its partial inhibitory activity (Fig. 1B), however, no further elevation in Tyr-416 phosphorylation (~2-fold) was detected in NbE cells after EGF treatment. Taken together, these data indicated that DOC-2/DAB2 could directly suppress the Tyr-416 phosphorylation, but not Tyr-527 phosphorylation, of c-Src via binding to the SH3 domain of c-Src.

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

The effect of DOC-2/DAB2 on the phosphorylation status of Erk induced by c-Src was also studied in NbE cells. As shown in Fig. 6B, EGF induced an approximately 4-fold elevation of Erk phosphorylation in NbE cells in the absence of peptide.
However, PPQ could further enhance the EGF-mediated Erk2 phosphorylation (13-fold). Using the control peptide LLQ, we observed a moderate induction of EGF-mediated Erk2 phosphorylation, because LLQ was a partial antagonist (Fig. 1B). These data provide further evidence that DOC-2/DAB2 is involved in the negative feedback mechanism modulating c-Src activity via the interaction between proline-rich domain and SH3 domain in prostatic epithelia.

**DISCUSSION**

From our previous studies (5–7), apparently, DOC-2/DAB2 protein can suppress the signal cascade elicited by mitogens via multiple pathways. To delineate the mechanism of action of DOC-2/DAB2 in more detail, we analyzed its interactive protein in this study. From a pull-down assay (Fig. 1A), in addition to Grb2, we further found that Src family proteins were able to interact with the C terminus, but not the N terminus, of DOC-2/DAB2. However, it is interesting to notice that the N terminus of DOC-2/DAB2 contains a disabled motif as in DAB1 phosphorylated by c-Src on its tyrosine residue (15, 16). The phosphorylated DAB1 protein serves as a binding site for SH2 domain binds to phosphorylated Tyr-527 (C terminus). The active Src is illustrated by the presence of phosphorylated Tyr-416, non-phosphorylated Tyr-527, and two intramolecular interactions critical for maintaining the closed conformation. The SH3 domain binds to the kinase linker connecting the SH2 domain to the kinase domain. The SH2 domain binds to phosphorylated Tyr-527 (C terminus). The active Src is illustrated by the presence of phosphorylated Tyr-416, non-phosphorylated Tyr-527, and both relaxed intramolecular interactions. The proline-rich domain of DOC-2/DAB2 can interlock the SH3 domain of Src that leads to the suppression of Src kinase activity. Y, non-phosphorylated tyrosine; "Y", phosphorylated tyrosine

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

**Acknowledgments**—We thank Gina Hernandez for excellent technical assistant, Andrew Webb for editorial assistance, and Dr. Koenneman for reading this manuscript. All GST-SH3 fusion plasmids and Src expression vector were kindly provided by Dr. Bing Wang.

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The Role of DOC-2/DAB2 in Signal Cascade

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J. Biol. Chem. 2003, 278:6936-6941. doi: 10.1074/jbc.M210628200 originally published online December 8, 2002

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