DCC (deleted in colorectal cancer) is a candidate tumor suppressor gene. However the function of DCC remains elusive. Previously, we demonstrated that forced expression of DCC induces apoptosis or cell cycle arrest (Chen, Y. Q., Hsieh, J. T., Yao, F., Fang, B., Pong, R. C., Cipriano, S. C. & Krepelat, F. (1999) Oncogene 18, 2747–2754). To delineate the DCC-induced apoptotic pathway, we have identified a protein, DIP13α, which interacts with DCC. The DIP13α protein has a pleckstrin homology domain and a phosphotyrosine binding domain. It interacts with a region on the DCC cytoplasmic domain that is required for the induction of apoptosis. Although ectopic expression of DIP13α alone causes only a slight increase in apoptosis, co-expression of DCC and DIP13α results in a ~5-fold increase in apoptosis. Removal of the DCC-interacting domain on DIP13α abolishes its ability to enhance DCC-induced apoptosis. Inhibition of endogenous DIP13α expression by small interfering RNA blocks DCC-induced apoptosis. Our data suggest that DIP13α is a mediator of the DCC apoptotic pathway.

The candidate tumor-suppressor gene deleted in colorectal cancer (DCC)1 was first cloned from a locus on chromosome arm 18q where allelic deletions occur in over 70% of primary colorectal tumors (1). Since that time, loss of heterozygosity at the DCC locus and loss of DCC expression have been shown in many other tumor types (2) including prostate carcinomas (3). Since that time, loss of heterozygosity at 18q has been reported that apoptosis induced by DCC is different from that by death receptor/caspase-8 pathway or the mitochondria-dependent pathway (18). Here we report the identification of DCC-interacting protein 13α (DIP13α) and present evidence showing that this DCC partner may serve as an adaptor mediating the DCC apoptotic signal.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF247338.

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The abbreviations used are: DCC, deleted in colorectal cancer; DIP, DCC-interacting protein; siRNA, small interfering RNA; oligonucleotide; RACE, rapid amplification of cDNA ends; Pipes, 1,4-piperazine diethanesulfonic acid; HA, hemagglutinin; EST, expressed sequence tag; BLAST, basic local alignment search tool.

MATERIALS AND METHODS

Cell Culture and Reagents—Colon adenocarcinoma cell line DLD1 was maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. All chemicals, unless otherwise specified, were obtained from Sigma-Aldrich.

Yeast two-hybrid Screen and Mapping—The LexA-based system (19) was used in our experiment. Because of a strong self-activation of the LexA-DCC cytoplasmic domain (1124–1447) bait, we deleted the last 44 amino acids of the DCC C terminus, which contains an acidic domain. This deletion eliminated its activation activity. We used this DCC bait to screen a cDNA library constructed from HeLa cells. We also used B42-DCC cytoplasmic domain (1124–1447) prey to screen a collection of 576 known bait proteins.

For mapping analysis, series of deletion mutants were constructed using either convenient restriction sites or PCR with appropriate primers. These mutants were assayed in the yeast system for interaction. Additional cDNA Cloning and Sequence Analysis—A 5′-RACE kit (Invitrogen) was used to clone the additional 5′ sequence. cDNA was reverse-transcribed from a total RNA sample prepared from HeLa cells using a gene-specific primer (5′-TCGGATTCCTGCTATACGAAAGATGTTAT-3′). Another gene-specific primer (5′-GAACAGGGATCCTGGA-
CAATAAAAATA-3') was used in RACE PCR together with anchor primer obtained from the manufacturer. The 5'-RACE product was sequenced.

An open reading frame was identified and translated into its corresponding protein using Lasergene software (DNA* Inc.). Protein features were analyzed using the NCBI BLAST service (www.ncbi.nlm.nih.gov/) and the GCG Molecular Sequence Analysis Package (Accelrys Inc.).

Intron-exon junctions were mapped by aligning cDNA sequence with its corresponding genomic sequences in the Human Genome Project database (www.ncbi.nlm.nih.gov/genome/guide/human/).

Construction and Expression of Fusion Proteins—All cDNA fragments were generated by PCR using high fidelity polymerases (Invitrogen) and cloned into an entry vector using the gateway cloning system (DIP13). Internal deletion of DIP13 (DIP13Δ428–530) was generated by digesting DIP13 ORF with Bam HI and XmaI, blunting the ends, and ligating. Expression vectors used were pDEST-TO-HisFlag (N-Flag tag) and pDEST27 (N-GST tag). Transient transfection of DLD1 cells was performed using LipofectAMINE Plus (Invitrogen) according to the manufacturer's instruction.

Antibodies—Anti-DCC, anti-HA, and anti-Flag antibody was purchased from PharMingen, CLONTECH and Sigma-Aldrich, respectively. Rabbit anti-DIP13 was made commercially using two peptides located at its C terminus and linked by 3 glycines (SSRPNQASSEggg).

Western Blotting and Immunoprecipitation—Two days after transfection, cells were suspended in lysis buffer (10 mM Pipes, pH 7.0, 0.5% Nonidet P-40, 80 mM KCl, 20 mM NaCl, 1 mM MgCl2, 5 mM EDTA, 1 mM dithiothreitol, and protease inhibitors) and subjected to repeated freeze-thaw cycles. For Western blotting, protein extracts were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with the indicated antibodies. Target proteins were visualized using the enhanced chemiluminescent method. For immunoprecipitation, lysates were incubated overnight with the indicated antibodies. Immune complexes were precipitated with protein A-Sepharose beads and washed with lysis buffer before being resolved on SDS-PAGE.

Transfection—Plasmid DNA was transfected with LipofectAMINE Plus (Invitrogen) according to the manufacturer's protocol.

Cell Death Analysis—Forty-eight hours after transfection, floating and attached cells were collected and enumerated by the trypan blue exclusion method. The results obtained from this method correlate well with other standard apoptosis assays used previously (9).

RNA Interference—siRNA for DIP13α and DIP13β (as a specificity control) were synthesized by Dharmacon Research Inc. The siRNA duplexes for alpha and beta, respectively, are as follows (Sequences 1 and 2).

**GUG CAG CAA CAC ACC UGA C dTdT**
ddT/CAC GUC GUU GUG UGG ACU G

**Sequence 1. Alpha duplex.**

**GCA GCA CCU CCC CCU U dTdT**
ddT/CGU CGU GGA GAG GAG GGA A

**Sequence 2. Beta duplex.**

Cells were transfected with RNA duplex and DCC expression vector using Oligofectamine (Invitrogen) following the manufacturer’s protocol. Cells were recovered for Western blotting or cell death analysis 48 h post-transfection.

Microinjection—Plasmid DNA and siRNA were injected into the nucleus of HeLa cells using an Eppendorf microinjector. Injections were performed with each plasmid at a concentration of 125 ng/ml. Duplex siRNA oligos were injected at a concentration of 400 pM, resulting in an estimated delivery of 5–20 molecules/cell. An average of 200 cells was injected per condition. To quantify cell death, healthy living fluorescent cells were counted 1–2 h after microinjection, and the percent survival was determined—24 h post-injection.

RESULTS

Identification of DCC-interacting Proteins—We have previously shown that the DCC cytoplasmic domain is required for the induction of apoptosis (9). To identify potential DCC signaling mediators, we used the DCC cytoplasmic domain (1124–1447) as bait to isolate interacting proteins with the yeast two-hybrid system. Because the LexA-DCC bait protein has strong activation activity, we deleted the last 44 amino acids of the DCC C terminus, which contains an acidic domain. This deletion eliminated its activation activity. We used this DCC bait to screen a cDNA library constructed from HeLa cells. From this screening, we identified the human sina-1, sina-2, proteasome subunit p40, and six novel genes that we named DIPs (DCC-interacting proteins). In addition, the DCC cytoplasmic domain (1124–1447) fused with the transcriptional activator B42 was used as prey to screen a collection of 576 known proteins. From this screening, we identified several proteins that interact with the DCC cytoplasmic domain in yeast, among them the Dro sophila seven-in- absentia (Sina) and human FKBP12 protein. In total, 13 DCC-interacting proteins were identified. Fig. 1 shows the specific interaction of the DCC cytoplasmic domain with several selected DIPs.

Mapping of Interaction Domains—We mapped the interactors of DCC with DIP13α and DIP13β using the two-hybrid system. The DCC–DIP13α interaction domain located at its C terminus and linked by 3 glycines (SSRPNQASSEggg). The DCC–DIP13β interaction domain coincided with the region (amino acids 1240–1273) coincided with the region (amino acids 1240–1273) with the region (amino acids 1240–1273) that is required for DCC to induce apoptosis (10). Therefore, our subsequent studies were focused on DIP13α. The DCC-interacting domain on the DIP13α was also mapped. This fragment (amino acid 454–646) contains a phosphotyrosine binding domain (Fig. 2B).

In Vivo Interaction between DCC and DIP13α—To validate the results obtained from the yeast two-hybrid system, DCC-DIP13α interaction was confirmed in mammalian cells by co-immunoprecipitation. Human colon adenocarcinoma DLD1 cells were transiently transfected with DCC and DIP13α, and...
cell lysates were prepared 24 h post-transfection. When DIP13α was immunoprecipitated from the lysates by affinity-purified antibody to DIP13α, a protein of ∼190 kDa was co-precipitated and recognized by an antibody specific to DCC on immunoblot (Fig. 3). Reciprocally, anti-HA antibody was able to immunoprecipitate HA-DCC and co-precipitate full-length DIP13α (Fig. 3).

DIP13α cDNA and Its Genomic Structure—The DIP13α cDNA obtained from the yeast two-hybrid screening was considered partial because the AUG codon was not in a Kozak consensus context. A BLAST search of the human EST data base also predicted a longer gene for DIP13α. An additional 1.6-kb 5′ sequence was subsequently cloned via a 5′-RACE experiment, which identified a start codon in a good Kozak consensus context. Altogether, a 3042-base contiguous cDNA sequence was obtained, which encodes a protein of 709 amino acid residues (GenBank™ accession number AF424738). The DIP13α protein contains a pleckstrin homology (PH) domain (20), a phosphotyrosine binding (PTB) domain (21, 22), and a coiled-coil domain (23) that partially overlaps with the PTB domain (Fig. 2B). The DIP13α cDNA is identical to the AKT2-interacting gene, APPL (24), with the exception of a shorter 3′-untranslated region. Alignment of the cDNA sequence with the corresponding genomic sequence in the Human Genome Project data base predicted that the DIP13α gene has 22 exons spanning an ∼36-kb genomic sequence. All intron-exon junctions conform to the GT-AG rule.

DIP13α Enhances Apoptosis Induced by DCC—To explore the functional relevance of DIP13α to the DCC apoptotic pathway, we transiently expressed the full-length DIP13α in DLD1 and 293T cells. We have previously shown that expression of DCC induces apoptosis in DLD1 as demonstrated by DNA fragmentation and caspase-3 activation (9). As shown in Fig. 4A, expression of DCC doubled the apoptotic population compared with vector control. This ratio of cell death was consistent with the results obtained with 293T cells (10, 18). Expression of DIP13α alone slightly increased the number of dead...
Mediation of DCC-induced apoptosis by DIP13α. A, DLD1 cells were transfected with vector, DIP13α, or DCC alone or a combination of DCC and DIP13α. Successful expression of transfected genes was confirmed by Western blotting. Apoptotic cells were enumerated as the percentage of dead cells to the total number of cells. B, DLD1 cells were transfected with different vectors either alone or in combination. The number of apoptotic cells was determined. Three independent experiments were performed. Error bars represent standard deviations.

FIG. 5. Requirement of the endogenous DIP13α in DCC-induced apoptosis. siRNA oligos (30 μl of 20 μmol oligo/60-mm plate) were transfected together with either control vector or DCC vector (4 μg/60-mm plate) into DLD1 cells. Cells were recovered 48 h post-transfection and used for Western blotting (A) or trypan blue exclusion assay (B). Three independent experiments were performed, each in triplicate. A combination of either control or DCC vector or DIP13α siRNA together with green fluorescent protein (GFP) vector was injected into cells, and an average of 200 cells were injected per condition in each experiment. Three independent experiments were performed (C).

Discussion

Although DCC was cloned in 1990, its function is still largely unknown. Recently, we and others have shown that DCC expression induces apoptosis and/or cell cycle arrest (9, 10). To delineate the signaling mechanism of DCC-induced apoptosis, we identified several known and novel molecules that interact specifically with the DCC cytoplasmic domain. The known molecules include Drosophila sina, human Sina-1 (Siah-1), Sina-2 (Siah-2), proteasome subunit p40, and FKBP12 protein. Siah-1 has been shown to regulate DCC protein level via the ubiquitin-proteasome pathway (25). It is possible that Siah-2 and proteasome subunit p40 have a similar effect on DCC, whereas the consequence of FKBP12-DCC interaction is unclear. Among the 13 DCC-interacting molecules identified, only DIP13α interacts with the region of DCC that is required to induce
apoptosis. In the present study, we have shown that DIP13α interacts specifically with DCC in yeast and mammalian cells, that expression of DIP13α enhances DCC-induced apoptosis, that removal of the DCC-interacting domain of DIP13α abolishes DIP13α’s ability to enhance DCC-induced apoptosis, and that reduction of endogenous DIP13α expression blocks DCC-induced apoptosis. These results indicate that DIP13α is a mediator of the DCC apoptotic signal. It is noteworthy that we observed the interaction between exogenous DCC (transfection with an expression vector) and exogenous DIP13α or exogenous DCC and endogenous DIP13α in DLD-1 and 293 mammalian cells by immunoprecipitation-Western blot. However, we were not able to perform an endogenous DCC and endogenous DIP13α interaction experiment due to the fact that most of cell lines do not express endogenous DCC. Interestingly, the neuroblastoma cell line, IMR32, expresses a high level of endogenous DCC but fails to undergo apoptosis. We were not able to detect DCC-DIP13α interaction by immunoprecipitation-Western blot in IMR32 cells under our experimental conditions. The lack of DCC-DIP13α interaction may explain why DCC does not induce apoptosis in IMR32 cells.

Recently a gene named APPL, which interacts with AKT2, has been identified (24). DIP13α is identical to the APPL gene. AKT is a serine/threonine kinase that can inhibit apoptosis (26). Because DIP13α/APPL can interact with AKT, we wondered whether DCC-induced apoptosis is mediated by DIP13α through the interference of the AKT function. Although AKT1, -2, -3 proteins were detected in DLD1 cells, AKT1 was more prominent than the other two (data not shown). We did not detect any changes in the total amount of AKT protein nor the phosphorylation status of threonine 308 and serine 473 of AKT in DLD1 and 293 cells expressing DIP13α and DCC (data not shown). Therefore, the role of AKT in DCC-induced apoptosis remains elusive.

Interestingly, Forcet et al. (18) reported that DCC induces cell death independently of either the mitochondria-dependent pathway or the death receptor/caspase-8 pathway. Moreover, DCC interacts with both caspase-3 and caspase-9 and drives the activation of caspase-3 through caspase-9 without a requirement for cytochrome c or Apaf-1. Combined with our data, this suggests that DCC, DIP13α, and caspase-9 may coordinate DCC-induced apoptosis. It was shown that amino acids 1243–1264 on DCC were required and sufficient for DCC to induce apoptosis (10). It appeared that DCC-(1243–1264) was also required for interaction with caspase-9 (18) and for interaction with DIP13α (the present study). Our data further indicated that the DCC-interacting domain of DIP13α (DIP13α(454–646)) was necessary for DCC to induce apoptosis. The DIP13α(454–646) fragment can have a significant effect on apoptosis when co-expressed with either full-length DCC or DCC-(1240–1273) (Fig. 4B). Deletion of the DCC-interacting domain on DIP13α (DIP13αΔ428–530) abolished its ability to enhance DCC-induced apoptosis. Namely, expression of DCC and DIP13αΔ428–530 induced the same level of apoptosis as DCC alone (Fig. 4, A and B). The apoptosis that occurs in cells expressing DCC alone was most likely caused by DCC interaction with the endogenous DIP13α in DLD1 cells. Indeed, reduction of endogenous DIP13α expression blocked DCC-induced apoptosis. Experiments are under way to determine the relationship between DIP13α and caspase-9 in DCC-induced apoptosis.

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