Rho GTPase-Activating Protein Deleted in Liver Cancer Suppresses Cell Proliferation and Invasion in Hepatocellular Carcinoma

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Abstract
Depleted in liver cancer (DLC1) is a candidate tumor suppressor gene recently isolated from human hepatocellular carcinoma. Structurally, DLC1 protein contains a conserved GTPase-activating protein for Rho family protein (RhoGAP) domain, which has been thought to regulate the activity of Rho family proteins. Previous studies indicated that DLC1 was frequently inactivated in cancer cells. In the present study, we aimed to characterize the tumor suppressor roles of DLC1 in hepatocellular carcinoma. We showed that DLC1 significantly inhibited cell proliferation, anchorage-independent growth, and in vivo tumorigenicity when stably expressed in hepatocellular carcinoma cells. Moreover, DLC1 expression greatly reduced the motility and invasiveness of hepatocellular carcinoma cells. With RhoGAP-deficient DLC1 mutant (DLC1-K714E), we showed that the RhoGAP activity was essential for DLC1-mediated tumor suppressor function. Furthermore, the 292– to 648–amino acid region and the steriodogenic acute regulatory related lipid transfer domain played an auxiliary role to RhoGAP and tumor suppressor function of DLC1. Taken together, our findings showed that DLC1 functions as a tumor suppressor in hepatocellular carcinoma and provide the first evidence to support the hypothesis that DLC1 suppresses cancer cell growth by negatively regulating the activity of Rho proteins. (Cancer Res 2005; 65(19): 8861-8)

Introduction
Hepatocellular carcinoma is the fifth commonest cancer worldwide and is the second leading fatal cancer in Southeast Asia and Hong Kong. Etiologically, hepatocellular carcinoma is closely associated with chronic hepatitis B and C virus infection, cirrhosis, and aflatoxin B1 intake (1). However, the molecular mechanisms leading to the development and progression of hepatocellular carcinoma remain unclear. In hepatocellular carcinoma, deletions of chromosomal materials are common and of a nonrandom pattern, with recurrent deletions on chromosomes 1p, 4q, 8p, 13q, 16q, and 17p. These strongly suggest that putative tumor suppressor genes may be located in these chromosome arms (2–7).

Recently, a candidate tumor suppressor gene—frequently depleted in liver cancer (DLC1)—was identified in primary hepatocellular carcinoma sample (8) and mapped to 8p21.3-22, where chromosomal deletion is particularly common in human cancers (2, 9–11). DLC1 mRNA encodes a protein with 1,091 amino acid residues and is ubiquitously expressed in human tissues (8). The amino acid sequence of DLC1 shares 86% homology with rat p122RhoG and this strongly suggests that DLC1 is a p122RhoG human orthologue. p122RhoGAP was first identified as a phospholipase C-61 binding protein in a rat brain expression library screening (12). Structurally, both p122RhoGAP and DLC1 contain three conserved functional domains—sterile α motif (SAM), RhoGAP, and steriodogenic acute regulatory related lipid transfer (START) domains (13, 14). Proteins containing a RhoGAP domain usually function to catalyze the hydrolysis of GTP that is bound to Rho family proteins. When the bound GTP is hydrolyzed to GDP, Rho proteins return to the inactive basal state. Thus, RhoGAPs negatively regulate Rho-mediated cellular processes, such as actin cytoskeleton organization, gene expression, and cell cycle progression (15, 16).

Evidence has shown that hyperactivation of Rho proteins is oncogenic (17–20). Clinically, Rho proteins were found to be overexpressed in different human cancers and overexpression of Rho proteins was associated with more aggressive tumor behavior (21, 22). Several members of the RhoGAP family have been suggested to possess potential tumor suppressor function. For instance, antitumor function of p190RhoGAP has been shown by in vitro studies. Overexpression of antisense p190RhoGAP RNA is able to transform normal NIH3T3 fibroblasts. In contrast, overexpression of the wild-type p190RhoGAP can suppress v-Ha-Ras–induced transformation (23). Therefore, it is logical to hypothesize that DLC1 might have tumor suppressor functions by negatively regulating tumor cell growth or tumor progression through down-regulation of oncogenic activity of Rho family proteins.

In this study, we showed that DLC1 reduced the proliferation rate, anchorage-independent growth ability, and in vivo tumorigenicity of hepatocellular carcinoma cells. Stable expression of DLC1 also significantly suppressed the motility and invasiveness of hepatocellular carcinoma cells. Furthermore, DLC1 inhibited Rho-dependent stress fiber formation in fibroblasts and hepatocellular carcinoma cells, and this Rho inhibitory function was essential for the DLC1-mediated growth inhibition in hepatocellular carcinoma cells. Thus, our findings support the hypothesis that DLC1 is a RhoGAP antagonizing the oncogenic activity of Rho proteins and serves as a tumor suppressor gene in human hepatocellular carcinoma.
Materials and Methods

Cell lines. Human hepatocellular carcinoma cell lines, SMMC-7721 and BEL7402, which lack DLC1 expression, were obtained from Shanghai Institute of Cell Biology (14, 24). The murine fibroblast cell line Swiss-3T3 was obtained from American Type Culture Collection (Manassas, VA). The rodent fibroblast cell line Rat 6 was kindly provided by Dr. W.L. Hsiao (Hong Kong Baptist University, Hong Kong ref. 25). SMMC-7721 and BEL7402 were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics. Swiss-3T3 and Rat 6 cells were cultured in DMEM supplemented with 10% (v/v) calf serum and antibiotics at 37°C in a humidified incubator with 5% CO2.

Plasmid construction. A 3.5-kb fragment of full-length coding sequence of the DLC1 gene was amplified from normal liver tissue and cloned into pDNA31(−) (set 2, reverse). Subsequently, an aliquot of the first PCR products were used as templates for the second PCR, which was performed by DNA sequencing.

Mutagenesis. A DLC1 fragment (1-804 amino acids) was released from pDNA31/DLC1 plasmid by EcoRI digestion and subcloned into plasmid II SK(+)(set 1, reverse). The site-specific mutation of K714E was introduced into DLC1 cDNA by PCR mutagenesis with two consecutive PCR amplifications. For the first PCR amplification, PCR fragments contain DLC1-K714E mutation was generated from pSK+-/DLC1 (1-804 amino acids) with two sets of PCR primers: 5′-GGATGGATAGAGAAGCTG-3′ (set 1, forward), 5′-GATCATGGAATCTGCTGCTGAG-3′ (set 1, reverse), 5′-GTCGGAGGAGTATTTTCCCAGG-3′ (set 2, forward), and 5′-TATACGACTCACTAGGGG-3′ (set 2, reverse). Subsequently, an aliquot of the first PCR products were used as templates for the second PCR, which was designed to amplify the full-length insert with the desired mutation in the full-length amplified fragment. The final amplified product was excised and gel purified. The NcoI/BamHI fragment of DLC1 cDNA in pSK+-/DLC1 (1-804 amino acids) plasmid was then replaced by this PCR-amplified fragment. The full-length DLC1-K714E mutant was generated by releasing the full-length DLC1-K714E fragment (1-804 amino acids) from pSK+-/DLC1 K714E plasmids by EcoRI digestion and subcloned into pcDNA3.1/DLC1 plasmid. The DNA sequences and the reading frame of the recombinant plasmids were confirmed by DNA sequencing.

Establishment of DLC1 stably expressing cells. pcDNA31(−)/DLC1 plasmid (2 μg) was transfected into SMMC-7721 cells with FuGene 6 Transfection Reagent as described by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). After 24 hours, the transfected cells were trypsinized and split onto 100-mm culture dish at a density of 1 x 10⁴ per dish. Transfected cells were selected for 2 weeks in culture medium containing 0.75 mg/mL G418 (Invitrogen, Carlsbad, CA). Single G418-resistant colonies were isolated from the culture dish using a cloning cylinder (Bellco Biotechnology, Vineland, NJ). The expression of DLC1 was determined by reverse transcription-PCR (RT-PCR) and Northern blotting as described previously (14).

Cell proliferation assay. Cells (2.5 x 10⁵) were plated in each well of 12-well culture plates containing 1 mL DMEM supplemented with 10% or 0.5% FBS. Cells were harvested at 24-hour intervals for 7 days. Cell numbers were counted by trypan blue exclusion assay with hemocytometer. The doubling time of proliferating cells was determined by nonlinear regression (exponential growth) model using GraphPad Prism version 3.00 (GraphPad Software, San Diego, CA).

Anchorage-independent growth assay in soft agar. Agarose culture medium (0.5%, 5 mL) containing 10% FBS was used to coat the bottom of each of the wells of the six-well culture plates. After hardening, 1 x 10⁴ cells were suspended in agarose culture medium (0.3%, 2 mL) containing 10% FBS warmed at 4°C and plated onto the bottom layer. The cell solution was allowed to set for 30 minutes at room temperature before moving into the 37°C CO₂ incubator. Colonies (≥10 cells) formed in the soft agarose culture were counted and photographed 2 weeks after inoculation.

Cell motility assays. For cell motility assay, 8 x 10⁴ cells were seeded onto 60-mm dishes and grown for 24 hours. Cells were treated with 10 μg/mL mitomycin C (Sigma, St. Louis, MO) for 3 hours before the wounding. A linear wound was made by scraping a pipette tip across the confluent cell monolayer. Cells were rinsed with PBS and grown in DMEM supplemented with 10% FBS for additional 48 hours. The cell motility in terms of wound closure was measured by photographing at three random fields at the time of wounding (time 0) and at 24 and 48 hours after wounding.

In vitro cell invasion assay. For in vitro cell invasion assay, 3 x 10⁴ cells were suspended in 300 μL serum-free DMEM and loaded onto the upper compartment of invasion chamber that contained a polycarbonate membrane with an 8-μm pore size and coated with a layer of extracellular matrix (ECM; Chemicon International, Temecula, CA). After 60 hours of incubation, the invasive cells that had migrated through the ECM layer to the complete medium in the lower compartment were stained, and the numbers of invasive cells were photographed and counted under the microscope.

In vivo tumorigenicity assay. SMMC-7721 and SMMC-7721/DLC1 cells were harvested and resuspended in PBS. Cells (1 x 10⁶) were inoculated s.c. into the right flank of 6-week-old female BALB/c nude mice using a 25-gauge needle (5 mice for each group). Tumor size was monitored weekly by measure the largest and smallest diameters of tumor and estimated according to the formula: volume = 1 / 2 x (largest diameter) x (smallest diameter). This experiment was preformed following the Animals (Control of Experiments) Ordinance (H.K.) and Institute's guidance on animal experiments.

Immunofluorescence microscopy. Cells (5 x 10⁴) were seeded onto coverslips 16 hours before transfection. At 24 hours after transfection, the cells were serum starved for an additional 24 hours and treated with 200 ng/mL lysophosphatidic acid (LPA; Sigma) for 30 minutes. Cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 minutes. Fixed cells were blocked with 3% bovine serum albumin and incubated with 1:1,000 rabbit polyclonal anti-Myc antibody (A14; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours followed by 1:100 FITC-conjugated goat anti-rabbit antibody (Amersham, Piscataway, NJ) and 1:1,000 rhodamine-conjugated phalloidin (Sigma) for 1 hour with extensive washing in between. Cells were counterstained with 4,6-diamidino-2-phenylindole (Calbiochem, San Diego, CA) and mounted in Vectashield antifade mountant (Vector Laboratories, Burlingame, CA). Images were captured under magnification of x1,000 by a fluorescence microscope equipped with a charge-coupled device camera (Leica, Wetzlar, Germany).

Coloncy suppression assay. Cells (2 x 10⁵) were seeded onto 35-mm dish 1 day before transfection. Each of the various pcDNA3.1/DLC1 constructs (2 μg) or pcDNA3.1/DLC1/K714E empty vector was cotransfected with 0.2 μg pBABE-puromycin selection vector into the cells using FuGene 6 Transfection Reagent. After 24 hours, cells were seeded onto 10-cm culture dishes in 1.5 dilution and grown in culture medium containing 0.6 μg/mL puromycin (Sigma) for 2 weeks. The puromycin-resistant colonies were fixed with 3.7% formaldehyde, and the colony formation efficiency was examined by Giemsa staining (Sigma).

Results

DLC1 inhibited cell proliferation, anchorage-independent growth, and tumorigenicity of hepatocellular carcinoma cells. We have shown previously that ectopic overexpression of DLC1 cDNA can suppress cancer cell growth in colony suppression assay (26). To further characterize the tumor suppressor function of DLC1 in hepatocellular carcinoma, in this study, we established a cellular model in which DLC1 cDNA was stably expressed in SMMC-7721 cells that lack endogenous DLC1 expression. The ectopic expression of DLC1 in stably transfected cells was confirmed by semiquantitative RT-PCR and Northern blot analysis.
DLC1 suppressed cell motility and invasiveness of hepatocellular carcinoma cells. To test whether DLC1 affects the motility of hepatocellular carcinoma cells, wound healing assay was done on SMMC-7721 and SMMC-7721/DLC1 cells in the absence or presence of a cell division inhibitor, mitomycin C. Our results showed that the motility of DLC1 stably expressing cells was significantly reduced. Similar results were also obtained when the cells were pretreated with mitomycin C, which indicated that the reduction of cell motility in DLC1-expressing cells was not due to the difference in proliferation rate (Fig. 3A). Consistently, the cell motility of SMMC-7721/DLC1 was significantly reduced in cell motility assay using invasion chamber without ECM coating, which confirmed that expression of DLC1 inhibited cell motility (Supplementary Fig. S1). Furthermore, we asked whether DLC1 could suppress the invasiveness of hepatocellular carcinoma cells. To this end, we did in vitro cell invasion assay using invasion chamber coated with an ECM layer (27). In sharp contrast with the parental SMMC-7721 cells, the number of invasive cells was dramatically reduced in DLC1 stably expressing cells (Fig. 3B). Thus, our results clearly showed that DLC1 suppressed the cell motility and invasiveness of hepatocellular carcinoma cells.

Expression of DLC1 inhibited Rho-mediated stress fiber formation. The existence of RhoGAP domain on DLC1 protein strongly suggests that DLC1 may function as a negative regulator toward the activity of Rho family proteins. Previously, we have shown that bacterially expressed DLC1 protein significantly enhanced the intrinsic GTP hydrolysis activity of two well-characterized Rho family proteins, RhoA and Cdc42 (14). This observation implied that DLC1 facilitated the conversion of the active GTP-bound Rho proteins to the GDP-bound inactive form. It is well known that Rho proteins play a critical role in regulating cytoskeleton organization and mediate actin stress fiber formation upon extracellular stimulations (28, 29). Actin stress fibers are disassembled in serum-starved cells but restored by LPA treatment through activation of Rho proteins. In this study, we further tested the in vivo RhoGAP activity of DLC1 by transiently expressing Myc-tagged DLC1 cDNA in hepatocellular carcinoma cell lines and fibroblast cell lines. The effects of DLC1 on the activity of Rho proteins were examined under fluorescence microscope with regard to morphologic changes and actin stress fiber formation. As shown in Fig. 4B, LPA induced the formation of actin stress fibers in serum-starved SMMC-7721 cells. However,
in DLC1-overexpressing cells, the formation of actin stress fibers was significantly suppressed. Furthermore, overexpression of DLC1 resulted in extensive cell rounding and cortical retraction. It is of note that these morphologic changes were remarkably similar to that caused by the Rho inhibitor, C3 exoenzyme, in HeLa cells (30), suggesting that these morphologic changes were due to inhibition of Rho proteins. Consistent observation was obtained from both hepatocellular carcinoma cell lines SMMC-7721 and BEL7402 and fibroblast cell lines Swiss-3T3 and Rat 6, suggesting that the effects of DLC1 on actin stress fiber formation and cell morphologic changes were not cell line–specific response.

In silico analysis had revealed that several lysine and arginine residues are highly conserved in various mammalian RhoGAPs. A previous study on the rat p122RhoGAP (DLC1 orthologue) has provided evidence that Lys706 residue is involved in stimulating the GTP hydrolysis of active GTP-bound Rho proteins (13). To further confirm the RhoGAP function of DLC1, we constructed a RhoGAP-deficient mutant by converting the Lys714 into glutamate (K714E), which corresponds to the Lys706 in rat p122RhoGAP. Expression of DLC1-K714E mutant abolished the DLC1-induced cell rounding and cortical retraction. In addition, on LPA induction, DLC1-K714E mutant-expressing cells formed actin stress fibers as efficiently as the empty vector control (Fig. 4B). Similar findings were also reproduced in another DLC1-null hepatocellular carcinoma cell line (BEL7402) as well as in fibroblast cell lines (Swiss-3T3 and Rat 6; data not shown). Taken together, our results strongly suggest that DLC1 functions as a RhoGAP and negatively regulates the activity of Rho proteins in vivo.

RhoGAP activity was essential for DLC1-mediated tumor suppressor function. We hypothesized that DLC1 suppressed cancer cell growth through negatively regulating the activity of Rho proteins. To test this hypothesis, colony suppression assays were done on SMMC-7721 cells transfected with wild-type and RhoGAP-deficient mutant DLC1 cDNA. We found that ectopic overexpression of wild-type DLC1 significantly suppressed the colony formation in SMMC-7721 cells (Fig. 5A). Indeed, this finding was highly consistent with that found in cancer cell lines of different tissue types as reported by us and others (26, 31, 32). In contrast to wild-type DLC1, expression of the RhoGAP-deficient mutant had no effect on the colony formation ability of SMMC-7721 cells. The number of colony formed by DLC1-K714E transfected cells was comparable with that of the empty vector (Fig. 5A). Thus, our data suggest that the RhoGAP activity is essential for DLC1-mediated tumor suppressor function and lend further support to the notion that DLC1 exerts
tumor suppressor function by negatively regulating the activity of Rho proteins.

Cooperation among the multifunctional domains of DLC1 was required for its growth inhibition of hepatocellular carcinoma cells. DLC1 contains three conserved functional domains. Apart from the RhoGAP domain, SAM and START domains are located at the NH2 and COOH termini of the DLC1 protein, respectively (Fig. 4A). We have shown in this study that the RhoGAP domain of DLC1 is essential for negative regulation of Rho activity and suppression of cancer cell growth. However, the roles of the SAM and START domains in DLC1 functions are largely unclear. To address this question, we expressed different truncated DLC1 cDNAs in SMMC-7721 cells (Fig. 4A). The expression of the truncated DLC1 proteins was confirmed by Western blotting using anti-Myc antibody (Fig. 5B). The RhoGAP activity and tumor suppressor function of these truncated constructs were determined by their ability to inhibit Rho-mediated actin stress fiber formation (Fig. 4B) and to suppress colony formation (Fig. 5A) in hepatocellular carcinoma cells, respectively. Because RhoGAP is essential for DLC1-mediated tumor suppressor function, we first asked whether the RhoGAP domain alone was sufficient to inhibit the activity of Rho proteins and suppress colony formation in hepatocellular carcinoma cells as efficiently as the wild-type DLC1. In this regard, we transiently expressed the DLC1 fragment containing the RhoGAP domain (DLC1-GAP) in SMMC-7721 cells. Surprisingly, the DLC1-GAP failed to induce morphologic change and inhibit actin stress fiber formation when overexpressed in hepatocellular carcinoma cells (Fig. 4B). Moreover, we did not find any significant difference in the colony formation ability between hepatocellular carcinoma cells expressing DLC1-GAP and those transfected with empty vector control (Fig. 5A). Similarly, the colony formation ability of hepatocellular carcinoma cells expressing DLC1 fragment containing either the SAM domain (DLC1-SAM) or the START domain (DLC1-START) alone was also comparable with that of the empty vector control (Fig. 5A). DLC1-SAM and DLC1-START also had no effect on hepatocellular carcinoma cell

Figure 4. Effect of DLC1 on cell morphology and Rho-dependent stress fiber formation. A, schematic representation of the various constructs of DLC1. The full-length DLC1, DLC1-FL (1-1,091 amino acids), contains three major functional domains—SAM, RhoGAP, and START. Various fragments of DLC1 cDNA were subcloned into Myc-tagged expression vector, pCS2+MT. +, significant inhibition of actin stress fiber formation or colony formation in hepatocellular carcinoma cells; −, no significant inhibition of actin stress fiber formation or colony formation in hepatocellular carcinoma cells. B, morphologic change and actin stress fiber formation of SMMC-7721 cells transfected with various DLC1 mutants. SMMC-7721 cells grown on glass coverslips were transfected with Myc-tagged DLC1 mutants or empty vector. Cells were serum starved for 18 hours and the formation of stress fibers was induced by LPA. The expression of DLC1 was determined by polyclonal anti-Myc antibody (A14) followed by FITC-conjugated goat anti-rabbit antibody. The actin stress fibers were stained with rhodamine-conjugated phalloidin. Arrowheads, cell expression of DLC1-FL and DLC1-START significantly inhibited stress fiber formation and induced cell rounding and cortical retraction.
pressed the NH2 terminus (of START domain in an important role in regulating DLC1 functions. To test the role SAM and RhoGAP (i.e., amino acid residues 292-639) may play DLC1 is not necessary for the RhoGAP and tumor suppressor function of

carcinoma cells (Fig. 5).

Significantly suppressed colony formation in hepatocellular carcinoma cells. Thus, cooperation among multifunctional domains in DLC1 is required for RhoGAP and tumor suppressor functions.

Next, we tested the RhoGAP and tumor suppressor functions of COOH-terminal (DLC1-CAV) and SAM domain–deleted (DLC1-ASAM) fragments of DLC1 in SMMC-7721 cells. COOH terminus of DLC1 showed no suppressive effects on the stress fibers and colony formation, whereas expression of DLC1-ASAM resulted in extensive cell rounding and cortical retraction accompanied with loss of actin stress fiber formation (Fig. 4B). In addition, DLC1-ASAM also significantly suppressed colony formation in hepatocellular carcinoma cells (Fig. 5A). These findings indicate that SAM domain is not necessary for the RhoGAP and tumor suppressor function of DLC1, whereas an unidentified functional domain located between SAM and RhoGAP (i.e., amino acid residues 292-639) may play an important role in regulating DLC1 functions. To test the role of START domain in DLC1-mediated functions, we further expressed the NH2 terminus (DLC1-NAC) and the SAM and START domain–deleted fragment (DLC1-ASAMSTART), respectively, in hepatocellular carcinoma cells. Like the COOH-terminal fragment, expression of the NH2-terminal fragment of DLC1 also had no effects on the cell morphology and colony formation efficiency of hepatocellular carcinoma cells. However, compared with DLC1-ASAM, further deletion of the START domain resulted in loss of the inhibitory effect on actin stress fiber formation and colony formation in hepatocellular carcinoma cells (Figs. 4B and 5A). These findings indicate that the START domain is indispensable for DLC1-mediated RhoGAP and growth inhibitory function. The concurrence of RhoGAP activity and growth inhibitory functions among these various truncated DLC1 constructs provides further evidence to support the notion that the RhoGAP activity is essential for DLC1-mediated tumor suppression (Fig. 4A). Similar results were also obtained when these experiments were done in BEL7402 and Rat 6 cell lines, indicating that these findings were not merely due to a cell line–specific phenomenon (data not shown).

Discussion

DLC1 is a candidate tumor suppressor gene recently isolated from PCR-based subtractive hybridization in human hepatocellular carcinoma (8). Follow-up studies indicate that inactivation of DLC1 is implicated in hepatocarcinogenesis (14, 26, 33, 34). Although somatic mutations in DLC1 gene are rare (14, 35, 36), loss of heterozygosity is present in approximately half of primary hepatocellular carcinomas (8, 14, 35). DLC1 mRNA expression is significantly down-regulated in primary hepatocellular carcinoma samples (14, 26). Earlier in vitro studies using hepatoma cell lines not expressing DLC1 have revealed that epigenetic alterations, including promoter methylation, are also responsible for DLC1 silencing in hepatocellular carcinoma (14, 34). In addition, methylation of the DLC1 promoter was detected in ~24% of primary hepatocellular carcinoma samples (14). These findings suggest that epigenetic silencing of DLC1 is not merely an artifact introduced by in vitro tissue culture but may substantially contribute to human carcinogenesis (14). The frequent inactivation of the DLC1 gene in hepatocellular carcinoma prompted us to further investigate its tumor suppressor functions. We showed previously that transfection of DLC1 cDNA into hepatocellular carcinoma cell lines resulted in inhibition of colony formation (26). Recently, inactivation of the DLC1 gene has also been observed in human breast, gastric, and lung cancers (31, 32, 37, 38), suggesting that DLC1 functions as a bona fide tumor suppressor gene. Although several lines of evidence support the tumor suppression role of DLC1, little is known about the functions of the DLC1 and how it is implicated in human carcinogenesis.

To further substantiate the tumor suppressor function of DLC1, we stably expressed DLC1 cDNA in SMMC-7721 cells. Expression of DLC1 cDNA significantly suppressed cell proliferation in both mitogen-dependent and mitogen-independent cultures. In addition, stable expression of DLC1 resulted in reduction of anchorage-independent growth capability. DLC1 expression also suppressed the tumorigenicity of SMMC-7721 cells in nude mice xenograft model. Consistent with our findings, Yuan et al. recently showed that introduction of the DLC1 cDNA into other cancer cell lines also abolished the in vivo tumorigenicity in nude mice (33, 38). The above findings, when taken together, strongly suggest that the DLC1 gene functions as a tumor suppressor in human carcinogenesis.

Our previous in vitro study indicated that DLC1 negatively regulated the activity of Rho family proteins by promoting the GTP

Figure 5. Tumor suppressor function of DLC1 mutants. A, colony suppression assays of SMMC-7721 cells transfected with DLC1 mutants. Columns, mean of three independent experiments; bars, SD. Similar findings were also obtained from another hepatocellular carcinoma cell line BEL7402 lacking endogenous DLC1 expression (data not shown). B, Western blotting and confirmed that all DLC1 mutants were at expected size and were expressed at similar level. NS, nonspecific bands.
hydrolysis (14). In the present study, we further showed that DLC1 expression could significantly suppress Rho-dependent actin stress fiber formation in hepatocellular carcinoma and fibroblast cell lines. Thus, our findings strongly imply that overexpression of DLC1 can inhibit the activity of Rho proteins and further support the notion that DLC1 functions as RhoGAP in vivo.

Cell motility is tightly regulated by the activity of Rho proteins through actin cytoskeletal rearrangements (29). Recently, a growing body of evidence has suggested that deregulation of Rho proteins are implicated in cancer cell invasion and metastasis. For instance, overexpression of wild-type RhoA in rat hepatoma cells induced cell invasion in both in vitro and in vivo conditions (39). On the other hand, dominant-negative RhoA (RhoA N19) was sufficient to inhibit the invasiveness of human prostate cancer cells (40). It has also been documented that the RhoA-specific inhibitor C3 exoenzyme greatly suppressed the cell motility of hepatocellular carcinoma cell lines, Li7 and KYN-2. In sharp contrast, the cell motility of these cell lines was strongly induced by LPA treatment (41). Based on the above evidence, we hypothesize that DLC1, a negative regulator of Rho proteins, will reduce cell motility. Indeed, our data clearly showed that expression of DLC1 significantly inhibited cell motility and invasiveness of hepatocellular carcinoma cells.

More than 30 potential downstream effectors of Rho family proteins have been identified (42). These effectors may be responsible for the different specific cellular functions mediated by the Rho proteins. ROCK is one of the best characterized downstream effectors, which selectively binds to active GTP-bound Rho proteins and regulates cell cytoskeletal reorganization through phosphorylating a wide range of cytoskeletal proteins (43, 44). Recent studies indicated that deregulation of Rho/ROCK signaling pathway is responsible for cancer invasion and metastasis (22, 41, 45). It is tempting to speculate that DLC1 suppresses cell motility and invasiveness through down-regulation of the Rho/ROCK signaling pathway. Further experiments are warranted to clarify the functional relationship between DLC1 and Rho/ROCK signaling pathway.

To further characterize the Rho inhibitory and tumor suppressor activity of DLC1, various DLC1 truncated mutants was generated. RhoGAP-deficient mutant abolished DLC1-mediated growth inhibition when expressed in hepatocellular carcinoma cells. Thus, our results have shown for the first time that the RhoGAP activity of DLC1 is essential for its tumor suppressor function. Surprisingly, ectopic expression of the DLC1 RhoGAP domain alone did not inhibit stress fiber and colony formation. These observations indicate that, apart from the RhoGAP domain, other functional domains of the DLC1 protein may also play some cooperative role in regulating the DLC1 function in vivo. Indeed, deletion of START domain of DLC1 resulted in impaired inhibition on actin stress fiber and colony formation, suggesting that the START domain may play an auxiliary role to DLC1 in inhibiting Rho activity and tumor cell growth. The exact function of START domain is largely unknown, but a previous study on the other START domain-containing proteins indicated that this domain might be involved in membrane targeting and lipid binding (46). We speculate that START domain in DLC1 may be responsible for targeting DLC1 to particular subcellular localization where its specific substrates locate. We also noticed that the expression of COOH terminus of DLC1 (648-1,091 amino acids), which contains intact RhoGAP and START domain, did not affect the actin stress fiber formation and was unable to suppress colony formation in hepatocellular carcinoma cells. However, DLC1-ASAM (292-1,091 amino acids) significantly inhibited the activity of Rho proteins and colony formation ability of hepatocellular carcinoma cells. This result strongly suggests that an unidentified functional domain located at 292 to 648 may be important for the DLC1-mediated RhoGAP and tumor suppressor functions. Thus, we propose that both RhoGAP and START domains and an unidentified domain located within the region of 292–648-amino acid residues are required for DLC1-mediated growth and stress fiber inhibition, and further investigations are warranted in this regard. Furthermore, similar with the RhoGAP-deficient mutant, we noticed a close association between RhoGAP activity and tumor suppressor function of various truncated DLC1 cDNAs. Thus, our results suggest that the tumor suppressor function of DLC1 is related to its Rho protein inhibitory activity.

In conclusion, our findings thus far have indicated that DLC1 significantly inhibited Rho-mediated actin stress fiber formation, and the RhoGAP activity of DLC1 was essential for tumor suppressor function. Expression of DLC1 suppressed cell proliferation, anchorage-independent growth, tumorigenicity, and invasiveness of hepatocellular carcinoma cells.

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