Interaction of Deleted in Liver Cancer 1 with Tensin2 in Caveolae and Implications in Tumor Suppression

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Abstract

Deleted in liver cancer 1 (*DLC1*) is a recently identified tumor suppressor gene frequently underexpressed in hepatocellular carcinoma (HCC). DLC1 encodes a Rho GTPase-activating protein domain that exhibits growth-suppressive activity in HCC cell lines. Our recent finding has revealed that inhibition of Rho-mediated actin stress fiber formation by DLC1 is associated with its growth inhibitory activity. In the present study, we identified tensin2 as the novel binding partner of DLC1. Tensin2 belongs to a new family of focal adhesion proteins that play key roles in cytoskeleton organization and signal transduction. Dysregulation of tensin proteins has previously been implicated in human cancers. Tensin2 is highly expressed in human liver. Introduction of tensin2 into HCC cell lines with low expression of tensin2 caused significant growth inhibition and induction of apoptosis. Tensin2 directly interacted with DLC1 *in vitro* and *in vivo*. Both proteins localized to punctate structures in the cytoplasm. Sequence analysis of DLC1 and tensin2 identified caveolin-1 binding motif in both proteins. *In vivo* immunoprecipitation study confirmed that both proteins indeed interacted with endogenous caveolin-1, which is the major structural component of caveolae. Our findings presented here suggest a new model for the action of DLC1 in hepatocytes, whereby DLC1-tensin2 complex interacts with Rho GTPases in caveolae to effect cytoskeletal reorganization. (Cancer Res 2006; 66(17): 8367-72)

Introduction

The candidate tumor suppressor gene, deleted in liver cancer 1 (*DLC1*), was isolated from human hepatocellular carcinoma (HCC; ref. 1) and was mapped to chromosome 8p21.3-22, a region thought to harbor tumor suppressor genes and recurrently deleted in HCC and other solid tumors (2). *DLC1* gene is widely expressed in normal human tissues, but frequent underexpression of DLC1, ranging from 30% to 70%, was detected in HCC and various cancer cell lines (3–5). The tumor-suppressive effect of DLC1 was observed by ectopic expression of DLC1 in cancer cell lines lacking endogenous DLC1 expression (3–6). A growing body of evidence supports the notion that DLC1 acts as a tumor suppressor gene but the underlying mechanism remains to be an area of investigation.

DLC1 encodes a protein containing three major functional domains [i.e., sterile α motif (SAM), Rho GTPase-activating protein (RhoGAP), and steroidogenic acute regulatory–related lipid transfer (START) domains]. DLC1 is the human homologue of rat p122-RhoGAP, the RhoGAP activity of which has been shown (7). RhoGAP serves to convert the active form of Rho to the inactive state. Rho proteins function as important regulators in remodeling of actin cytoskeleton, regulation of transcription, cell proliferation, metastasis, and tumorigenesis (8, 9). Recently, we have reported that the RhoGAP activity of DLC1 is associated with its growth-suppressive effect on HCC cell lines (10).

In the present study, we have done yeast two-hybrid library screening to identity tensin2 as the first and novel binding partner of DLC1. Tensin2, a focal adhesion protein, localized at the end of stress fibers that share extensive sequence homology with tensin1 (11). Growing evidence suggests that tensins are not only structural proteins but also represent a new family of proteins that act as an important link among extracellular matrix, actin cytoskeleton, and signal transduction and have been implicated in human cancers (12). We have examined the expression level of tensin2 in HCC cell lines and addressed its growth-suppressive effect on HCC cell lines. Identification of caveolin-binding motif in both DLC1 and its binding partner, tensin2, prompted us to question whether DLC1 and/or tensin2 localizes in caveolae. Understanding the subcellular localization of DLC1 and tensin2 might derive mechanistic insight into the tumor-suppressive role of DLC1 in liver.

Materials and Methods

Plasmid constructions. Various expression constructs using pAS2-1, pGADGH, pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA), PGEX2T (Amersham Pharmacia Biotech, Piscataway, NJ), pcDNA3.1 (Invitrogen, Carlsbad, CA), and pCS2+MT were prepared by standard molecular biology techniques and PCR amplification of the described fragments. pAS2-1-based Gal4 DNA-binding domain (BD) vectors were made, carrying DLC1 with the following amino acids: DLC1 1-1091, DLC1 1-595, DLC1 1-291, DLC1 292-1091, DLC1 648-1091, and DLC1 375-509. Gal4 activation domain (AD) vectors based on pACT2 were constructed, carrying tensin2 with the following amino acids: tensin2 1-1409, tensin2 1113-1365, and tensin2 1113-1276. Plasmid for bacterial expression of glutathione *S*-transferase (GST) fusion protein of DLC1 375-509 amino acids was made using pGEX2T. GFP-tagged expression vector, pEGFP-C1, carrying full-length tensin2, was constructed. Eukaryotic expression vectors for Myc-tagged proteins were derived from pCS2+MT and prepared as follows: DLC1 1-1091 (FL, full-length), DLC1Δtensin2-BD (without tensin2 binding domain, 375-509 amino acids), DLC1ΔCBM (mutated caveolin-1 binding motif), tensin2 (full-length), tensin2-SH2PTB (1135-1409 amino acids), and tensin2-PTB (1113-1409 amino acids).

Yeast two-hybrid library screening and binding assay. Library screening was done by sequential transformation of the yeast strain CG1945 first with the bait, DLC1 1-1091 pAS2-1 plasmid carrying full-length DLC1 fused to Gal4-BD, and subsequently with human liver cDNA library (BD Biosciences Clontech) constructed in the Gal4-AD plasmid, pACT2, by large-scale lithium acetate transformation protocol. The transformation

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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procedure was done as previously described (13). To map the binding regions between DLC1 and tensin2, panels of DLC1 deletion mutants were tested for their binding activity with tensin2, and vice versa.

Site-directed mutagenesis. Expression constructs of DLC1 and tensin2 with mutated caveolin-1 binding motif (CBM) were made by changing the aromatic residues of the caveolin-1 binding motif, ΨXΨXXXXΨ (Ψ is an aromatic residue), to alanine. Specific mutations in both constructs were introduced by three-step PCR. For constructing DLC1ΔCBM mutant, a DLC1 fragment (321-2735 nucleotides) was subcloned into pcDNA3.1 vector at *Eco*RI site. First PCR was done using primer set (T7 and ΔCBM-R: 5′-CATGGCCTTGGGCACGGCCGCGCTAGCACC-3′) and the second PCR was done using primer set (ΔCBM-F: 5′-GGTGCTAGCGCGGCCGTGCCCAAGGCCATG-3′ and BGH-R). The third PCR product amplified by T7 and BGH-R primers was used to replace the 321-2735 nucleotides of the wild-type DLC1 in pCS2+MT vector by *Eco*RI digestion.

Cell culture. HEK293 and human HCC cell lines, HepG2, BEL7402, and SMMC7721, were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin, and streptomycin at 37°C in humidified incubator with 5% CO_2 in air. HEK293 and HepG2 cell lines were obtained from American Type Culture Collection (Manassas, VA), whereas BEL7402 and SMMC7721 were obtained from Shanghai Institute of Cell Biology (14). Transfection of cells was done with Lipofectamine 2000 reagent according to the instruction of the manufacturer (Invitrogen).

GST pulldown assay. GST pulldown assay using GST or GST fusion protein of DLC1 375-509 amino acids was done as described (13). In brief, GST fusion proteins were incubated with Myc-tagged tensin2-PTB–expressing cell lysates and the bound proteins were detected with anti-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoprecipitation. To examine if DLC1 and tensin2 interact *in vivo*, HEK293 cells were transiently transfected with Myc-tagged tensin2 and DLC1. Cells were lysed and lysates were incubated with anti-Myc antibody, after which immunoprecipitates were collected with protein G-sepharose (Amersham Pharmacia Biotech). The immunoprecipitates were subjected to gel electrophoresis and Western blot analysis using anti-DLC1 (BD Biosciences) and anti-Myc antibodies. The technical details were described elsewhere (13). To study if tensin2 and/or DLC1 interacts with caveolin-1, Myc-tagged tensin2 or DLC1 was transfected into SMMC7721 in which caveolin-1 was highly expressed. Endogenous caveolin-1 was immunoprecipitated with anti-caveolin-1 antibody (BD Biosciences) and the interacting proteins were detected with anti-Myc antibody.

Immunofluorescent staining. For subcellular localization studies of tensin2 and DLC1, cells seeded on coverslips were transfected with GFP-tensin2 and Myc-tagged DLC1 plasmids. Transfected cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS. Cells were then blocked with 5% bovine serum albumin in PBS and incubated with anti-Myc antibody, followed by rhodamine-conjugated secondary antibody. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Calbiochem, San Diego, CA) and mounted in Vectashield antifade mountant (Vector Laboratories, Burlingame, CA). Images were captured by a fluorescence microscope equipped with charge-coupled device camera (Leica, Wetzler, Germany). The endogenous vinculin was visualized by anti-vinculin antibody (Sigma, St. Louis, MO), followed by rhodamine-conjugated secondary antibody.

Colony formation assay. BEL7402 and HepG2 cells were seeded at a density of 2×10^5 per 35-mm plate 1 day before transfection. Two micrograms of tensin2-pCS2+MT or control pCS2+MT were cotransfected with 0.2 μg pBABE-puro selection vector into cells. Twenty-four hours after transfection, cells were seeded onto 10-cm culture plates at 1:10 dilution and grown in culture medium containing 1 μg/mL puromycin (Invitrogen) for 2 weeks. The puromycin-resistant colonies were fixed with 3.7% formaldehyde and stained with Giemsa (Sigma).

Reverse transcription-PCR analysis. Total RNA was extracted from HCC cell lines and HCC tissue samples with TRIzol reagent according to the protocol of the manufacturer (Invitrogen). First-strand cDNAs were synthesized from 1 μg of total RNA using random hexamers with GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). Quality of cDNA was assessed by amplification of a 200-bp fragment of β-actin. PCR amplification of tensin2 cDNA was done using a set of primers (forward, 5′-ATGAAGTCCAGCGGCCCTGTG-3′, and reverse, 5′-AGGCAAGGCCTGACAGGCTGAAGT-3′) to give a product of 245 bp, which covered 136-381 nucleotides of tensin2 (GI:38787956). The reaction was done under the

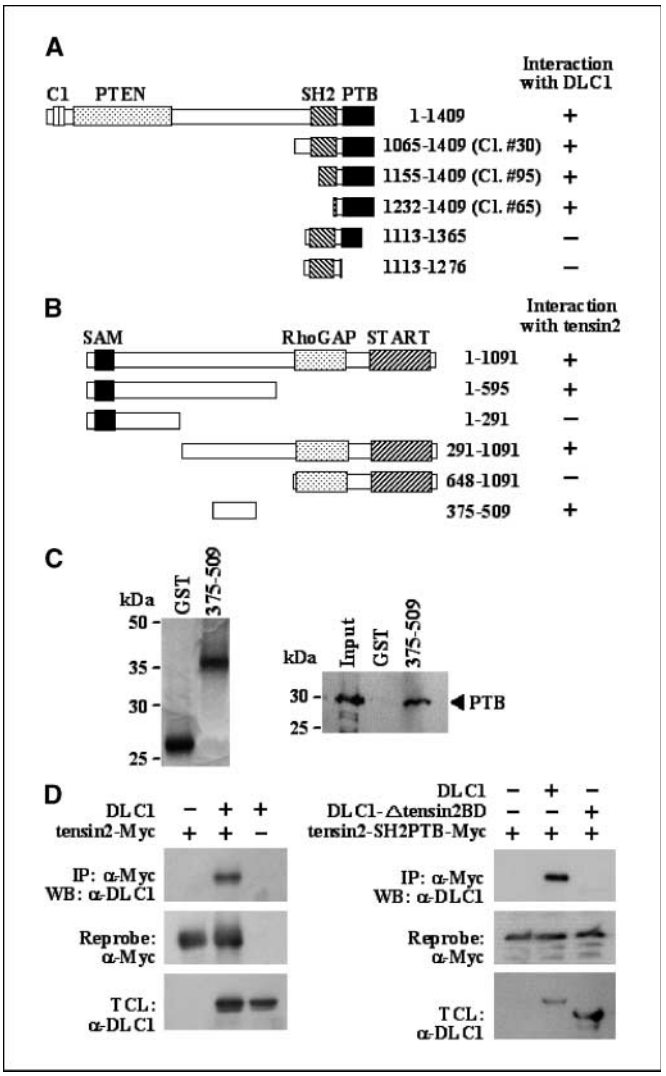

Figure 1. Interaction between DLC1 and tensin2. *A,* yeast two-hybrid transformation assay of tensin2 deletion clones with full-length DLC1. Tensin2 is a 1409-amino-acid protein composed of a PKC C1 domain at the NH_2 terminus followed by a PTEN region, and SH2 and PTB domains at the COOH terminus. These domains are indicated by patterned boxes. Clones 30, 65, and 95 encoding the COOH terminus of tensin2 were positive clones obtained from yeast two-hybrid library screening. Deletion mutant of tensin2, encoding 1113-1365 and 1113-1276 amino acids, did not interact with DLC1. Clone 65 encoding the PTB domain is the shortest positive clone interacting with DLC1. *B,* yeast two-hybrid transformation assay of tensin PTB domain with deletion mutants of DLC1. *Black, dotted,* and *hatched boxes,* structural domains of DLC1 SAM, RhoGAP, and START, respectively. The minimal interacting region of tensin2 located at 375-509 amino acids of DLC1. *C,* GST pulldown assay. HEK293 total cell lysate expressing Myc-tagged tensin2-PTB domain was pulled down by GST-DLC1 (375-509 amino acids of DLC1) fusion protein but not by the control GST protein (*right*). Bound protein was detected with anti-Myc antibody. *Input,* 10% of total cell lysate used in the assay. The GST and GST-DLC1 fusion protein proteins fractionated on gel were stained with Coomassie blue (*left*). *D, in vivo* interaction of DLC1 and tensin2. Lysates of HEK293 cells transfected with Myc-tagged full-length tensin2 with or without full-length DLC1 were subjected to immunoprecipitation (*IP*) with anti-Myc antibody followed by anti-DLC1 and anti-Myc immunoblotting (*WB*). Total cell lysate (*TCL*) was probed with anti-DLC1 antibody (*left*). Deletion of tensin2-interacting region in DLC1Δtensin2-BD abrogated its interaction with the COOH-terminal tensin2 mutant, tensin2-SH2PTB-Myc (*right*).

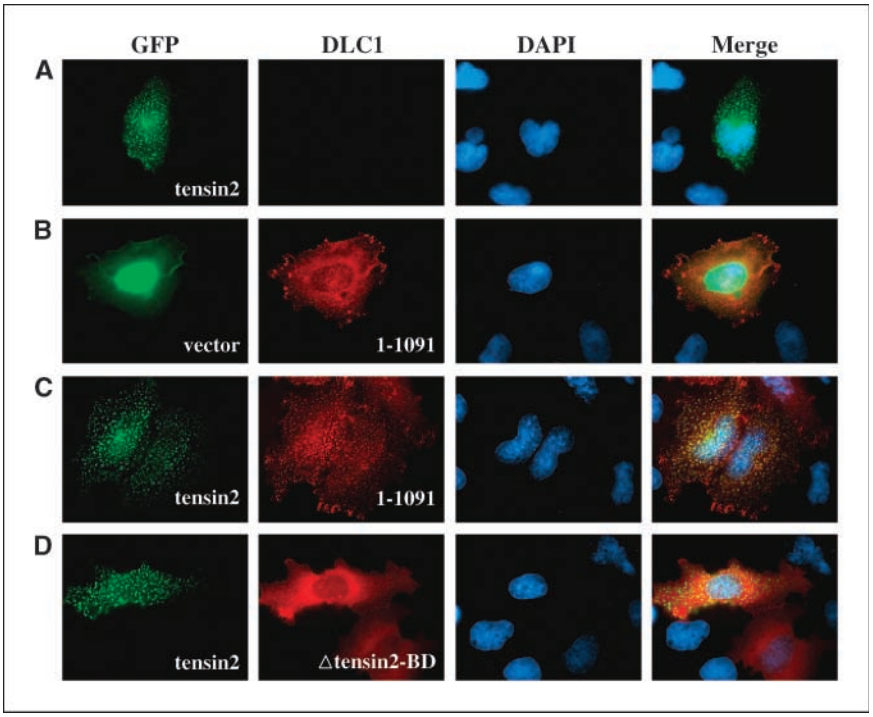

Figure 2. Subcellular colocalization of DLC1 and tensin2. SMMC7721 cells were transiently transfected with GFP-tagged tensin2 and Myc-tagged DLC1 constructs. DLC1 was visualized by anti-Myc antibody, followed by rhodamine-conjugated secondary antibody. Nucleus was visualized by DAPI staining. Overlays of GFP, rhodamine, and DAPI stained images are shown in the last column. *A* and *B,* subcellular localization of GFP-tagged tensin2 and Myc-tagged DLC1 1-1091 with GFP control vector. Tensin2 displayed a characteristic dotted focal adhesion pattern and DLC1 showed a scattered fine speckles pattern in the cytoplasm. *C,* coexpression of tensin2 and DLC1 showed colocalization of tensin2 with DLC1 and displayed an overlapping dotted pattern in yellow. Tensin2 interacted with DLC1. *D,* tensin2 did not colocalize with DLC1 mutant DLC1Δtensin2-BD, in which the tensin2 binding domain was deleted. The tensin2 binding domain is required for the subcellular localization of DLC1.

following condition: 95°C for 12 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds followed by a final extension at 72°C for 5 minutes.

Flow cytometry. BEL7402 cells were transfected with GFP control or GFP-tensin2 expression vectors. Forty-eight hours after transfection, cells were washed twice in cold PBS and subjected to cell sorting by flow cytometry. Sorted cells were fixed with cold 80% ethanol and stained with propidium iodide. DNA profile of cell populations was determined by flow cytometry analysis.

Luciferase assay. HEK293 cells in 24-well plates were transfected with different combinations of plasmids using FuGENE 6 reagent (Roche, Indianapolis, IN). Plasmids used included pCS2+MT-DLC1, pCS2+MT-DLC1Δtensin2-BD, pCS2+MT-tensin2, pUSE-RasQ61L (Upstate Biotechnology, Inc., Lake Placid, NY), pGal4-luc, pSRE-Luc reporter constructs (Stratagene, La Jolla, CA), and an internal control pRLSV40. Total amount of expression vectors was equalized with the empty vector. Twenty-four hours after transfection, cell lysates were harvested, prepared, and assayed using Dual Luciferase Reporter assay system (Promega, Madison, WI) according to the instructions of the manufacturer. Transfection efficiency was normalized with the Renilla luciferase activity. Each transfection was done in triplicates and two independent experiments were done. Data were expressed as means with SDs.

Results and Discussion

Identification of tensin2 as the binding partner of DLC1. To gain more insight into the molecular basis of DLC1 tumor suppressor and its role in hepatocarcinogenesis, we attempted to search for potential binding partners of DLC1 protein. We did yeast two-hybrid screening of a human liver cDNA library using the full-length DLC1 as bait. A total of 3×10^7 transformants were screened and positive clones obtained were confirmed by β-galactosidase activity. Sequence analysis showed that three of the positive clones (clones 30, 65, and 95) were partial clones with incomplete 5′ end and displayed identical nucleotide sequence to the 3′ end of tensin2 cDNA (GI:38787956; Fig. 1*A*). Tensin2 is dominantly expressed in liver, heart, kidney, and skeletal muscle (11). Structurally, tensin2 is the only tensin family member harboring a protein kinase C (PKC) C1 domain at the NH_2-terminal. It also possesses a PTEN (phosphatase and tensin homologue deleted from chromosome 10) homology region followed by src homology 2 (SH2) and phosphotyrosine binding domain (PTB) at the COOH terminal. All tensin family members share extensive sequence homology and are structurally related. Focal adhesion localization is the common feature of all members. We first made a full-length tensin2 construct and confirmed its interaction with DLC1 by yeast two-hybrid cotransformation assay (Fig. 1*A*). Previous study has shown that phospholipase C-δ1 interacts with the rat homologue, p122RhoGAP (15). In this study, we report tensin2 as the first and novel binding partner of the human homologue DLC1. Our findings raised an interesting question about how an adhesion protein, known to be intimately associated with cytoskeleton, might contribute to the tumor-suppressive role of DLC1. This prompted us to elucidate the functional significance of their interaction.

PTB domain of tensin2 interacts with the central region of DLC1. All three positive clones obtained from library screening encoded only the COOH-terminal region of tensin2 where SH2 and PTB domains are located. The smallest partial clone of tensin2 that interacted with DLC1 was clone 65, encoding tensin2 1232-1409 amino acids in which only the intact PTB domain was encoded (Fig. 1*A*). To confirm that PTB domain is the minimal region interacting with DLC1, we constructed two tensin2 deletion constructs with disrupted PTB domain, which carry 1113-1365 and 1113-1276 amino acids fused with Gal4-AD, and found that both constructs were unable to interact with DLC1 Gal4-BD plasmid. This suggests that the PTB domain of tensin2 is likely to be the minimal interacting domain for DLC1. On the other hand, to map the interacting region for tensin2 of DLC1, we generated various deletion mutants of DLC1 constructed in Gal4-BD plasmid and tested for interaction with tensin2-PTB domain (clone #65). Among the constructs tested, those possessing the central region of

DLC1 interacted with tensin2 (Fig. 1*B*). The minimal interacting region for tensin2 was mapped to 375-509 amino acids of DLC1. We then did *in vitro* GST pulldown assay and confirmed that the interaction between tensin2 and DLC1 was mediated by the determined minimal interacting regions. Specific binding with Myc-tagged PTB domain of tensin2 was observed with the GST-DLC1 fusion protein containing the region 375-509 but not with GST protein alone (Fig. 1*C*).

Tensin2 interacts and colocalizes with DLC1 *in vivo*. Due to the lack of antibodies to endogenous tensin2, we could only address the *in vivo* interaction by epitope-tagged tensin2. DLC1 and Myc-tagged tensin2 were transiently transfected into HEK293 cells. Results from the coimmunoprecipitation assay indicated that Myc-tagged tensin2 formed a complex with DLC1 in the transfected cells (Fig. 1*D*). The interaction between tensin2 and DLC1 was mediated by the COOH terminus of tensin2 and the deduced tensin2 interacting region of DLC1. The interaction was lost in mutant DLC1, DLC1Δtensin2-BD, in which the determined tensin2 interaction region was deleted. In light of this, we next sought to determine the subcellular compartments in which DLC1 interacts with tensin2. Towards this end, we expressed GFP-tagged tensin2 and Myc-tagged DLC1, either alone or together, in SMMC7721 cells. Ectopic expression of tensin2 in transfected cells displayed a scattered dotted pattern typical of a focal adhesion protein (Fig. 2*A*) whereas DLC1 displayed a cytoplasmic localization with fine speckles (Fig. 2*B*). Simultaneous expression of tensin2 and DLC1 gave an overlapping staining pattern of both proteins (Fig. 2*C*). However, tensin2 did not colocalize with the DLC1 mutant, DLC1Δtensin2-BD, in which the tensin2 interacting region was deleted (Fig. 2*D*). Moreover, the DLC1Δtensin2-BD mutant lost the fine patches pattern and displayed a uniform cytoplasmic staining. The result obtained from the DLC1 mutant suggests that the tensin2 interacting region is required for targeting DLC1 to a particular subcellular localization, whereby it might interact with other proteins. Similar observation was seen in other HCC cell lines, BEL7402 and HLE, as well as in Rat6 fibroblasts (data not shown). Our observation supported the direct physical interaction between DLC1 and tensin2 and suggested the ability of tensin2 in directing the subcellular localization of DLC1. Previous findings have shown that tensin2 localizes at the end of actin stress fibers and colocalizes with vinculin at focal adhesions (11) whereas DLC1

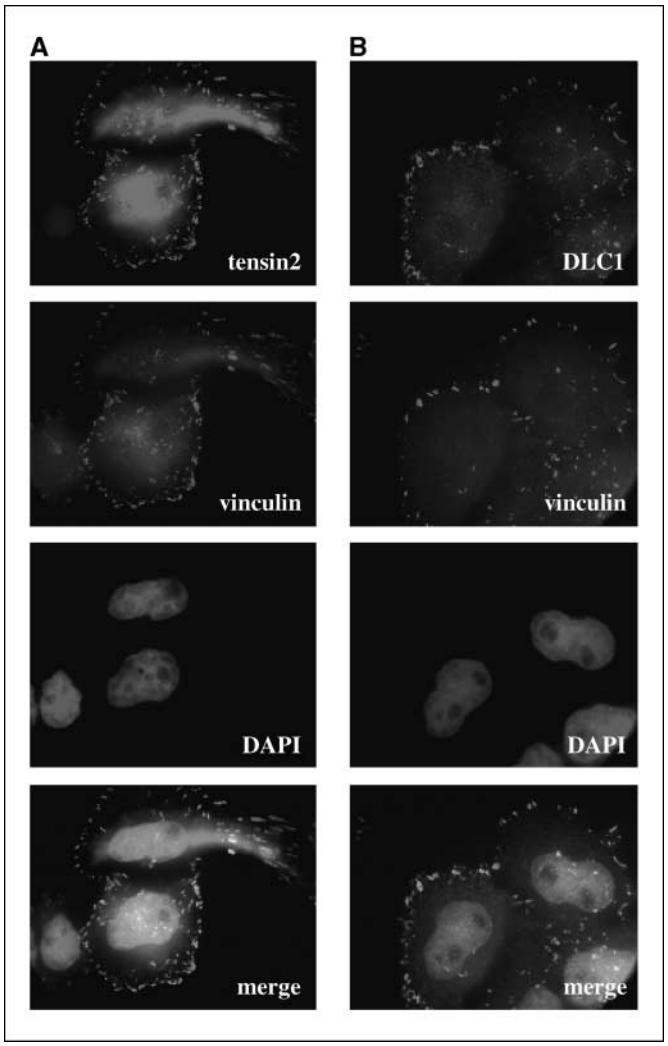

Figure 3. Tensin2 and DLC1 colocalized with vinculin. Myc-tagged tensin2 (*A*) and Myc-tagged DLC1 (*B*) were transiently expressed in SMMC7721 cells. Myc-tagged proteins were visualized by anti-Myc antibody, followed by FITC-conjugated secondary antibody. Endogenous vinculin was stained by anti-vinculin antibody, followed by rhodamine-conjugated secondary antibody.

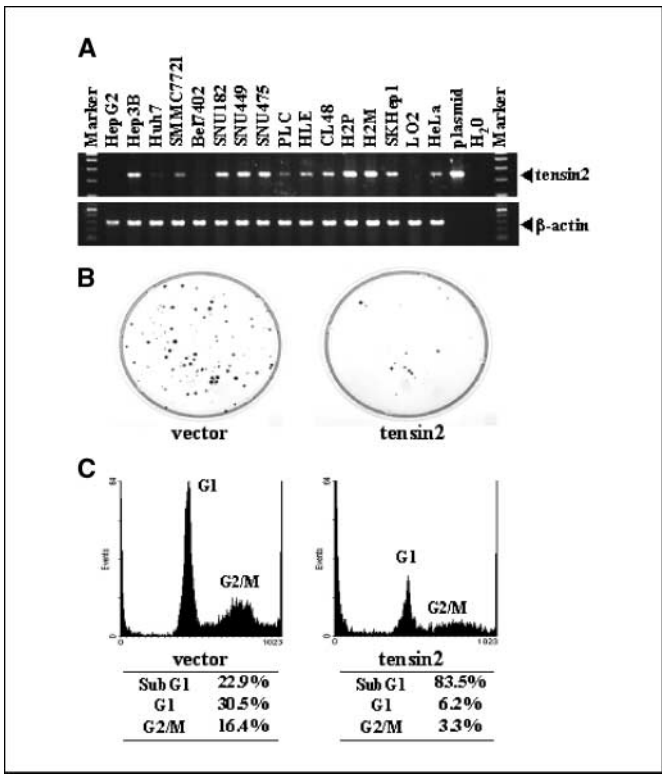

Figure 4. Growth inhibition and induction of apoptosis of HCC cell lines by tensin2. *A,* expression of tensin2 in HCC cell lines. Expression of tensin2 was determined by RT-PCR. PCR amplification of tensin2 cDNA was done using a set of primers flanking 1022-1233 nucleotides of tensin2. Tensin2 was not detected in three tested cell lines (HepG2, BEL7402, and LO2). Quality of RNA was determined by amplification of β-actin. *B,* colony formation assay. HCC cell line BEL7402, which lacks tensin2 expression, was transfected with tensin2 expression vector. Dramatic inhibition of colony formation was observed in tensin2-transfected cells as compared with the vector control. *C,* induction of apoptosis by expression of tensin2. Flow cytometry analysis was done to determine the DNA content of tensin2-transfected cells after staining with propidium iodide. Decrease in G_1 and G_2-M populations and increase in sub-G_1 population were observed in tensin2-transfected as compared with the vector control.

is present in the cytoplasm (6), and the rat homologue p122 RhoGAP colocalizes with vinculin at focal adhesions (16). Epitope-tagged tensin2 and DLC1 were expressed in SMMC7721 cells and were found to colocalize with the endogenous vinculin (Fig. 3).

Growth inhibition of HCC cell lines by tensin2. Tensin family members, tensin1 and cten, have been implicated in cellular transformation. Expression of human tensin1 was down-regulated in prostate and breast cancer cell lines (17) whereas the prostate-specific cten level was reduced in prostate cancer samples (18). The high expression level of tensin2 in liver tissue and its interaction with DLC1 prompted us to determine the expression of tensin2 transcript in HCC cell lines by reverse transcription-PCR (RT-PCR). Among the tested HCC cell lines, tensin2 mRNA was not detected in HepG2 and BEL7402 cells and was underexpressed in Huh7, SMMC7721, and PLC cells (Fig. 4*A*). We also examined the expression level of tensin2 in one nontumorigenic immortalized liver cell line, MIHA (19). Comparing with Hep3B, which has a relatively high expression among the HCC cell lines being examined, MIHA showed an even higher expression level of tensin2 (Supplementary Fig. S2). We further characterized the effect of tensin2 in two HCC cell lines, HepG2 and BEL7402, which have barely detectable endogenous tensin2 expression. Expression of tensin2 significantly suppressed the colony formation of BEL7402 (Fig. 4*B*) and HepG2 (data not shown). Thus, tensin2 is another tensin family member implicated in human cancers. However, we failed to obtain stable clones of tensin2 in several attempts. This might be due to its pronounced growth-suppressive property. Furthermore, flow cytometry analysis showed that tensin2-transfected cells displayed a remarkable decrease in G_1 and G_2-M populations and an almost 4-fold increase in sub-G_1 population as compared with the vector control (Fig. 4*C*). It is known from previous study that restoration of DLC1 in HCC cell lines also induced apoptosis and inhibited cell growth (6). However, whether both proteins act synergistically through their interaction warrants further investigation. Interaction between DLC1 and tensin2 is of particular interest because both interacting partners exert growth-suppressive effect on HCC cell lines.

Tensin2-DLC1 interacts with caveolin-1. Previous studies have shown that the rat homologue of DLC1, p122/RhoGAP, shows GAP activity for Rho (7) and localizes in caveolae where it regulates reorganization of actin filaments (20). Caveolae are lipid-enriched plasma membrane invaginations that concentrate diverse regulatory proteins implicated in signal transduction and pathogenesis of various cancers (21). Based on the previous reports, we asked whether tensin2 and DLC1 complex localized in caveolae. Cellular fractionation of SMMC7721 cells expressing Myc-tagged tensin2 and DLC1 revealed that both proteins were present in the cytosolic and membrane fractions (Supplementary Fig. S3). Notably, most proteins targeted to caveolae interact with the caveolin-scaffolding domain of caveolin-1 through at least one caveolin-1 binding motif, ΨXΨXXXXΨ or ΨXXXXΨXXΨ, where Ψ is an aromatic residue and X is any amino acid (21). Caveolin-1 is the principal structural component of caveolae membrane. Coincidently, each of tensin2 and DLC1 possesses one caveolin-1 binding motif (Fig. 5*A*). The potential binding motif, FGYRAPGY, was located in the amino acid residues 666-673 of tensin2, whereas FSWAVPKF was found in the amino acid residues 617-624 of DLC1. We questioned whether tensin2 and/or DLC1 interacted with caveolin-1 by doing coimmunoprecipitation assay. Transient transfection of Myc-tagged tensin2 or DLC1 into SMMC7721 cells in which caveolin-1 is highly expressed confirmed that both tensin2 and DLC1 indeed interacted with endogenous caveolin-1 (Fig. 5*B*). Many receptors and signaling molecules have been identified within caveolae (i.e., Src-like kinase, Ras, G proteins, epidermal growth factor receptor, nitric oxide synthase, and Rho GTPases). Recently, we have shown that DLC1 suppressed the formation of stress fibers by its RhoGAP activity and this suppression was associated with the growth-suppressive activity of DLC1 in HCC cell lines (10). Based on the documentation that Ras mediates Rho GTPases in oncogenic transformation (22), we did reporter assay to investigate the effect of DLC1 and tensin2 on the serum response element (SRE), which is one of the Ras effectors (23). A dominant active Ras mutant, RasQ61L, efficiently activated SRE. Expression of either DLC1 or tensin2 alone could not inhibit or only slightly inhibited the activation of SRE. The effect of inhibition was enhanced when DLC1 and tensin2 were coexpressed. However, the inhibitory effect was lost when tensin2 was coexpressed with the tensin2 binding domain–deleted DLC1 mutant, DLC1ΔBD (Fig. 5*C*). Our results showed that DLC1 and tensin2 cooperatively inhibited Ras signaling based on their specific interaction. The inhibitory activity of DLC1-tensin2 complex was lost when the potential caveolin-1 binding motif in DLC1 was mutated (Fig. 5*D*). The interaction of DLC1 and tensin2 with caveolin indicated that caveolae are at least one of the physiologic compartments whereby

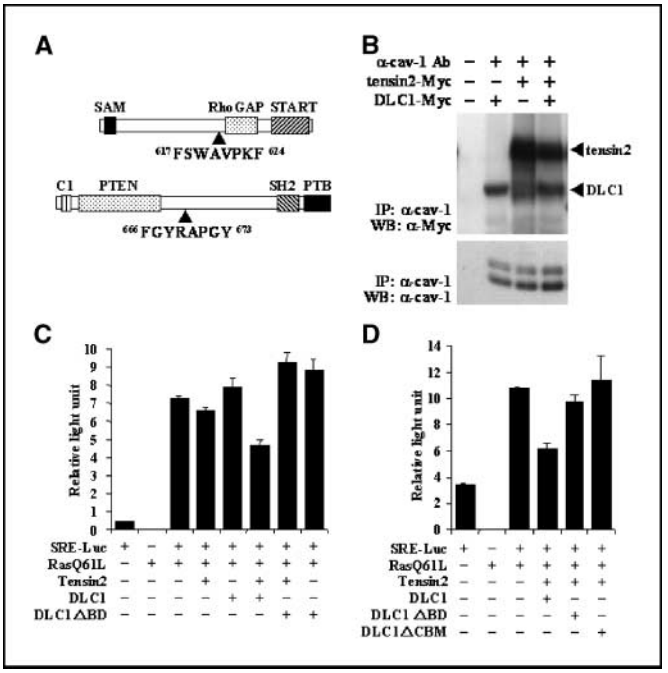

Figure 5. DLC1 and tensin2 localize in caveolae and have synergistic effect on Ras-mediated activation of SRE. *A,* caveolin-1 binding motif of tensin2 and DLC1. Schematic representations of tensin2 and DLC1. Putative caveolin-1 binding motifs are indicated. *B,* DLC1 and tensin2 interact with endogenous caveolin-1. Myc-tagged DLC1 and tensin2 were transfected into SMMC7721 cells in which caveolin-1 was highly expressed. Endogenous caveolin-1 was immunoprecipitated with anti-caveolin-1 antibody and immunoprecipitates were detected by immunoblotting with anti-Myc antibody. Both DLC1 and tensin2 interacted with endogenous caveolin-1. *C,* inhibition of Ras-mediated activation of SRE by cooperative effect of DLC1 and tensin2. Reporter assay done in HEK293 cells revealed that RasQ61L induced activation of SRE. DLC1 alone could not inhibit the activation whereas tensin2 displayed slight inhibition effect. The inhibition was enhanced when DLC1 and tensin2 were coexpressed. The inhibition was lost when tensin2 was coexpressed with DLC1ΔBD mutant in which the tensin2 binding domain was deleted. *D,* caveolin-1 interaction is required for the inhibition of SRE by DLC1-tensin2 complex. Inhibitory activity was abrogated when tensin2 was coexpressed with DLC1ΔCBM mutant in which the caveolin-1 binding motif was mutated.

DLC1 fulfills its growth-suppressive role. Because caveolae are rich in Rho family GTPases (24), DLC1 in caveolae likely acts through its RhoGAP activity to effect regulatory roles on actin cytoskeleton.

In this report, we have identified tensin2 as a novel interacting partner of DLC1. We have also established the growth inhibitory activity of tensin2 in HCC cell lines. In addition, we have also provided the first evidence of the subcellular localization of DLC1 and tensin2 in caveolae. We propose a new model for the action of DLC1 in which the formation of DLC1-tensin2 complex brings DLC1 in close proximity to Rho GTPases enriched in caveolae and facilitates the inactivation of Rho proteins through the RhoGAP activity of DLC1. Our results further corroborated our recent findings that DLC1 exhibits tumor-suppressive role by down-regulating Rho activity (10). Moreover, growing evidence has shown that caveolin-1 interacts with and compartmentalizes various signaling molecules which may sensitize cells towards apoptosis (21). Induction of apoptosis by DLC1 and tensin2 in connection with their caveolar localization warrants further investigation. Understanding the physiologic function and the significance of DLC1-tensin2 interaction in caveolae might provide insight into the molecular basis of the tumor-suppressive role of DLC1.

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