Mutations in the Focal Adhesion Targeting Region of Deleted in Liver Cancer-1 Attenuate Their Expression and Function

Yi-Chun Liao, Yi-Ping Shih, and Su Hao Lo

Center for Tissue Regeneration and Repair, Department of Biochemistry and Molecular Medicine, University of California, Davis, Sacramento, California

Abstract

Deleted in liver cancer-1 (DLC-1) is a RhoGTPase-activating protein (RhoGAP) domain containing tumor suppressor that is often down-regulated in various cancer types. Previously, we have shown that DLC-1 is recruited to focal adhesions by binding to the Src homology 2 domains of tensins and the focal adhesion localization is critical for the tumor suppression activity of DLC-1. To investigate whether mutations in the focal adhesion targeting (FAT) region might occur and attenuate the expression, localization, and function of DLC-1, we have first mapped the FAT region to the amino acid residues from 201 to 500, and then sequenced cDNAs and genomic DNAs encoding the FAT region from cancer patients. Several missense and nonsense mutations were detected. All missense mutations were further examined for the potential effect on the function of DLC-1. Although these mutations did not seem to affect the focal adhesion localization of DLC-1, the activities of suppressing tumor cell growth were impaired in two mutants: T301K and S308I. Consistent with the fact that the RhoGAP activity of DLC-1 is essential for inhibiting tumor cell growth, the RhoGAP activities were significantly reduced in these mutants, suggesting that the FAT region also contains a regulatory element for its COOH-terminal RhoGAP domain. Our studies have shown that mutations in DLC-1 may lead to loss of function and contribute to the tumorigenesis, and have revealed an allosteric regulation site for its RhoGAP activity. [Cancer Res 2008;68(19):7718–22]

Introduction

Tumor suppressors are genes that reduce the chance of normal cells turning into tumor cells. The loss of function of one or more tumor suppressor genes is believed to be an important step of cancer development. Deleted in liver cancer-1 (DLC-1) was originally isolated as a potential tumor suppressor gene often deleted in hepatocellular carcinoma (1). Further studies have indicated that down-expression of DLC-1 either by genomic deletion or promoter methylation is associated with a variety of cancer types including prostate, lung, breast, kidney, colon, uterus, ovary, and stomach (2, 3). Reexpression of DLC-1 in DLC-1 null cancer cell lines is able to suppress cell growth, induce morphologic change, decrease cell migration, and reduce tumorigenicity (2, 3). These data support a tumor suppressor role of DLC-1.

Human DLC-1 protein contains 1,091 amino acid (aa) residues sharing a sterile α motif (SAM) at the NH_2 terminus, a RhoGTPase-activating protein (RhoGAP) domain, and a steroidogenic acute regulatory–related lipid transfer (START) domain at the COOH terminus. The SAM domain is predicted to interact with proteins, RNA, or DNA (4), whereas the START domain may bind to lipid (5). The RhoGAP domain converts the active GTP-bound Rho protein to the inactive GDP-bound state and negatively regulates Rho GTPase. This RhoGAP activity is essential but not sufficient for many of the antioncogenic activities of DLC-1 (6–8). The COOH-terminal half of DLC-1 interacts with phospholipase C (PLC) δ1 and enhances the phosphatidylinositol 4, 5-bisphosphate–hydrolyzing activity of PLCδ1 (9). The COOH-terminal region also targets to caveolae presuming by interacting with caveolin-1 through several potential binding sites on DLC-1 (8, 10). However, the significance of caveolae localization of DLC-1 is not clear.

Recently, we have reported that the NH_2-terminal half of DLC-1 localizes to focal adhesions by binding to the Src homology 2 domains of tensins (11). This focal adhesion localization is required for the tumor suppression activity of DLC-1 because the tensin binding–defective DLC-1 mutants do not localize to focal adhesions and cannot suppress tumor cell growth (11, 12). Therefore, in addition to genomic deletion and promoter hypermethylation, mislocalization of DLC-1 protein may be another mechanism of acquiring tumorigenicity involving DLC-1 dysregulation.

In the present study, we report the analysis of the focal adhesion targeting (FAT) region, the discovery of mutations within the FAT region of the *DLC-1* gene, and the functional relevance of these mutations. Our finding further reveals a new allosteric regulatory site within the FAT region for the RhoGAP activity of DLC-1.

Materials and Methods

Mutation analysis. Prostate cDNA and colon genomic DNA samples were purchased from OriGene Technologies and BioChain, respectively. DNA fragments were amplified by PCR using DLC-1 primers 5′-GGCAGCCTGCCCTCTCCCAAGGAA (forward) and 5′-CAGGGCTGAGTCCGAATCTCCCTC-3′ (reverse). The PCR products were purified by QIAquick PCR purification kit (Qiagen) and then subjected to DNA sequencing. Samples that displayed mutations were subjected to another independent amplification and sequencing to rule out the PCR and DNA sequencing error. Mutations shown in this study were detected on both experiments.

Plasmid constructions and mutagenesis. The full-length and the region encoding residues 1 to 535 of human DLC-1 cDNA were constructed in the previous study (11). The truncated cDNA fragments encoding DLC-1 residues 279 to 535 and 201 to 500 were subcloned in frame into mammalian expression vector pEGFP-C2 (Clontech). The site-specific mutation of P290L, T301K, S308I, S320I, Y338L, Y442F, or K714E was introduced into DLC-1 cDNA by site-directed mutagenesis. All constructs were verified by DNA sequencing.

Cell culture, gene transfection, and colony formation assay. A549, HEK293T, and MDA-MB-468 cells purchased from American Type Culture

Y-C. Liao and Y-P. Shih contributed equally to this work.

Requests for reprints: Su Hao Lo, Center for Tissue Regeneration and Repair, Department of Biochemistry and Molecular Medicine, University of California, Davis, 4635 Second Avenue, Room 2000, Sacramento, CA 95817. Phone: 916-734-3656; Fax: 916-734-5750; E-mail: shlo@ucdavis.edu.

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Collection were cultured in DMEM supplemented with antibiotics and 10% fetal bovine serum. A549 and HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen), whereas MDA-MB-468 cells were transfected using SuperFect transfection reagent (Qiagen) according to the manufacturer's introductions. MDA-MB-468 cells were used for colony formation assay as previously described (11).

Immunofluorescence microscopy. A549 cells grown on glass coverslips were transfected and incubated at 37°C in 5% CO_2 for 16 h before microscopic imaging. Cells were fixed with methanol at −20°C. After rinsing with PBS, cells were incubated with 1:100 antitensin3 rabbit polyclonal antibody for 1 h. Samples were then incubated with 1:800 Alexa Fluor 594–conjugated secondary antibody (Molecular Probes) for 1 h and visualized with a Zeiss LSM 510 confocal microscope.

RhoA activity assay. RhoA activities were measured with the Rho Activation Assay Biochem kit (Cytoskeleton). In brief, 24 h after transfection, HEK293T cells were serum-deprived for another 24 h and then treated with lysophosphatidic acid (5 μmol/L; 30 min), which enhanced Rho-GTP levels. Cells were immediately washed with ice-cold PBS and lysed. Equal protein amounts of cell lysates were incubated with 30 μg glutathione *S*-transferase–Rho binding domain of Rhotekin (GST-RBD) beads for 1 h at 4°C. The beads were washed twice with washing buffer, and bound Rho proteins were analyzed by immunoblotting using an anti-Rho antibody (Cytoskeleton).

Results

Mutation analysis of the FAT region of DLC-1 from human patients. Recently, we have shown that the focal adhesion localization of DLC-1 mediated by the Src homology 2 domains of tensins is critical for the function of DLC-1 as a tumor suppressor (11). This finding has promoted us to investigate whether mutations occur in the FAT region and whether these mutations lead to mislocalization of DLC-1. If so, even a high level of DLC-1 transcript is detected, the expressed protein may not function appropriately. Previous studies had found that the NH_2-terminal half of DLC-1 localized to focal adhesion sites (11, 13). To further narrow down the region sufficient for the FAT of DLC-1, we had examined the subcellular localization of several green fluorescent protein (GFP)–DLC-1 fusion constructs. As shown in Fig. 1, although GFP–DLC-1 (1–535) and GFP–DLC-1 (201–500) colocalized with tensin3 at focal adhesions, GFP–DLC-1 (279–535) was diffused in the cytoplasm, demonstrating that the fragment 201 to 500 contains sufficient FAT sequences. With this information, we then focused our DLC-1 mutation analysis on human cDNA and genomic DNA encoding this region.

For mutation analysis, we have amplified the DNA fragment encoding the FAT region by PCR from 48 prostate cDNA samples [7 normal, 11 benign prostatic hyperplasia (BPH), and 30 cancers] for DNA sequence analysis. No mutation was found in any of normal prostate samples. We did not detect the PCR products in 1 of 11 BPH samples, and no mutation was detected in the remaining 10 BPH samples. Two of 30 prostate cancer samples did not produce PCR fragments, and 4 point mutations (1 nonsense and 3 missense mutations) were found in the remaining 28 cancer samples (Table 1). All mutations were reconfirmed by a second independent PCR amplification and DNA sequencing to eliminate potential PCR and sequencing error. Because the genomic DNAs of these samples were not available to us, we could not completely rule out the possibility that these mutations were derived during the reverse-transcription reaction. Therefore, we have analyzed genomic DNAs from 8 normal and 40 colon cancer samples. No mutation was found in normal samples; 3 cancer samples did not produce PCR fragments, and 4 mutations (1 nonsense and 3 missense mutations)

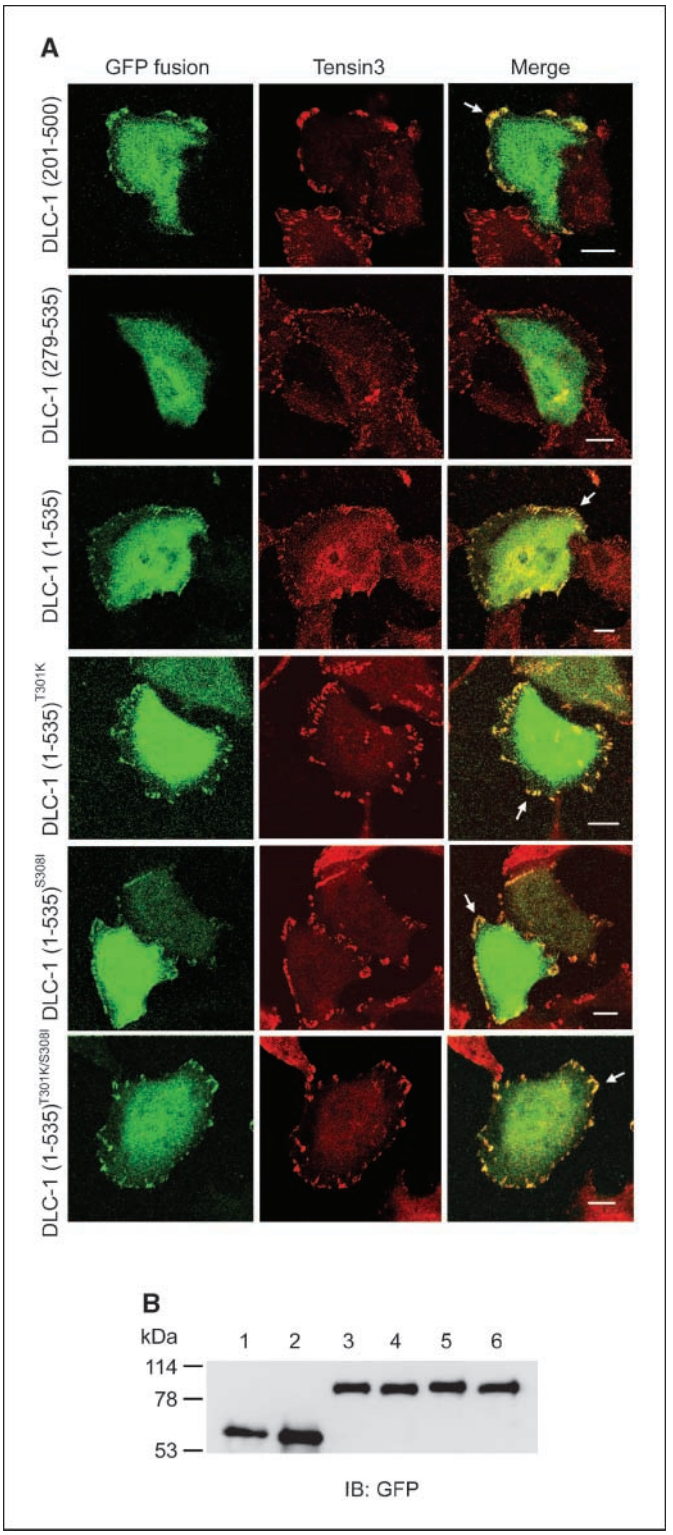

Figure 1. Identification of the FAT region of DLC-1 and focal adhesion localization of DLC-1 mutants. *A,* A549 cells grown on coverslips were transfected with indicated GFP-fusion constructs. After labeling with antitensin3 antibodies followed by Alexa Fluor 594–conjugated secondary antibody, cells were visualized with a confocal microscope. Arrows indicate GFP-fusion protein and tensin3 colocalized at focal adhesions. *Bar,* 10 μm. *B,* cell lysate from DLC-1 (201–500) (*lane 1*), DLC-1 (279–535) (*lane 2*), DLC-1 (1–535) (*lane 3*), DLC-1 $(1–535)^{T301K}$ (*lane 4*), DLC-1 $(1–535)^{S308I}$ (*lane 5*), or DLC-1 $(1–535)^{T301K/S308I}$ (*lane 6*) GFP fusion transfectant was immunoprecipitated with anti-GFP and analyzed by immunoblotting (*IB*) with anti-GFP to show similar expression levels and correct sizes of recombinant proteins.

Table 1. Summary of the mutation analyses within the FAT region of human DLC-1 in various tissues

In prostate (cDNA)	Nucleotide	Normal	BPH	Tumor	aa
Missense mutation	869 C > T	0	0	2 of 28	P290L
	902 C > A	0	0	1 of 28	T301K
	923 G > T	0	0	3 of 28	S308I
Nonsense mutation	929 C > A	0	0	1 of 28	S310Stop
Polymorphism	1060 G > A	7 of 7	5 of 10	12 of 28	V354M

In colon (genomic DNA)	Nucleotide	Normal	Tumor	aa
Missense mutation	923 G > T	0	1 of 37	S308I
	959 G > T	0	1 of 37	S320I
	1013 AC > TA	0	1 of 37	Y338L
Nonsense mutation	1016 T > A	0	1 of 37	L339Stop
Polymorphism	1060 G > A	2 of 8	6 of 37	V354M

were detected in the remaining cancer samples (Table 1). Meanwhile, the previously identified single nucleotide polymorphism (SNP) leading to V354M was found in our cDNA and genomic DNA mutation screening with a frequency of 35.6%.

The effect of mutations on the subcellular localization of DLC-1. The nonsense mutations (S310stop, L339stop) found in this study would result in truncated DLC-1 proteins containing only the first 309 or 338 of the 1,091 residues. Based on the known domain function, both mutant proteins would not contain the complete FAT, RhoGAP, and START domains. Hence, these truncated proteins would not be functional. To determine the effect of missense mutations on the subcellular localization of DLC-1, we have generated mutations in GFP–DLC-1 (1–535) expression constructs. We used this truncated DLC-1 fragment because the full-length DLC-1 often leads to cell morphology change due to its RhoGAP activity and it is difficult to observe the subcellular localization in rounded cells. We have examined P290L, T301K, S308I, S320I, and Y338L mutations and found all mutants localized to focal adhesions as effective as the wild-type (WT) fragment (Fig. 1; data not shown). Even the T301K/S308I double mutations localized to focal adhesions, indicating that at least mutations identified in this study did not significantly affect focal adhesion localization of DLC-1.

T301K and S308I mutations render the tumor suppression activities by reducing the RhoGAP activities. We further investigated the potential effects of these mutations in the full-length DLC-1 on their tumor suppression activities by colony formation assays. In agreement with previous studies, GFP–DLC-1^{WT} was able to suppress tumor cell growth and both Y442F (defective in tensin binding) and K714E (RhoGAP dead) mutants lost the suppression activities by showing >4.4 to 5.1 times more colonies than in GFP–DLC-1^{WT} (Fig. 2). Surprisingly, the suppression activities were significantly reduced in T301K, S308I, and T301K/S308I mutants (Fig. 2). Because both the RhoGAP activity and focal adhesion localization of DLC-1 are required for suppressing tumor cell growths (11, 12) and FAT is normal in these mutants (Fig. 1), we tested whether the RhoGAP activities were altered in these mutant cells. As shown in Fig. 3, the RhoA-GTP levels (the main target of DLC-1 RhoGAP domain) were significantly elevated in cells expressing GFP control, T301K, S308I, or T301K/S308I mutants comparing to the cells expressing DLC-1

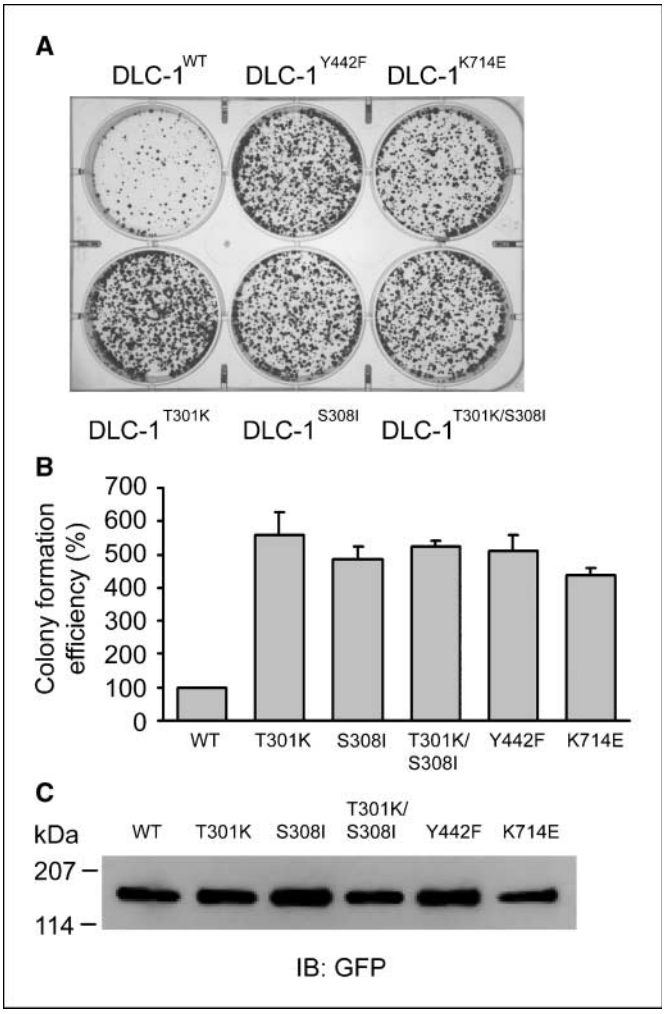

Figure 2. Colony formation assays of DLC-1 mutants. *A,* a representative colony formation assay is shown. MDA-MB-468 cells were transfected with indicated constructs. After being cultured in medium containing 0.8 mg/mL G418 for 2 wk, G418-resistant colonies were stained with crystal violet. *B,* the histogram shows the colony formation efficiency of each GFP–DLC-1 mutant comparing to the WT. *C,* cell lysate from each transfectant was immunoprecipitated with anti-GFP and analyzed by immunoblotting with anti-GFP to show similar expression levels and correct sizes of recombinant proteins.

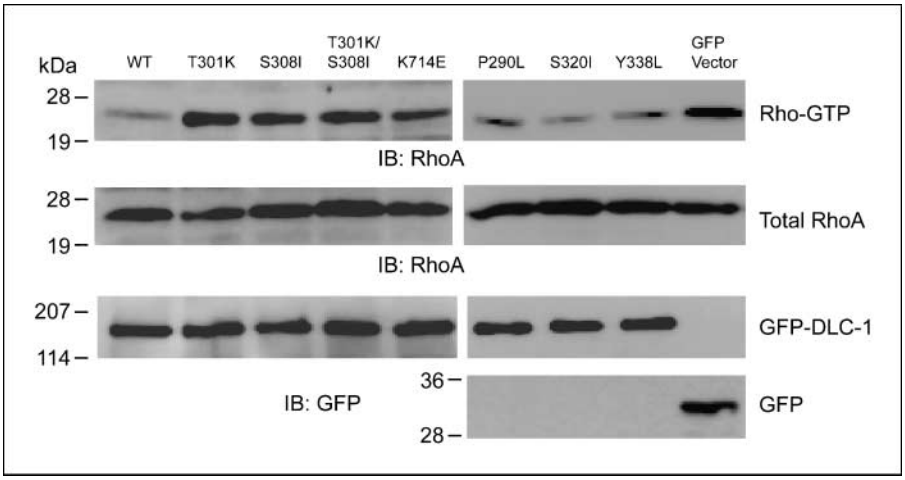

Figure 3. RhoGAP activities of DLC-1 mutants. HEK293T cells transfected with indicated GFP fusion constructs were treated with lysophosphatidic acid, and then RhoA-GTP levels were analyzed by GST-RBD pull down assay followed by anti-RhoA blotting (*top*). The expression levels of endogenous RhoA (*middle*) and recombinant GFP-fusion proteins (*bottom*) were confirmed by immunoblotting with anti-RhoA and anti-GFP antibodies.

WT, P290L, S320I, or Y338L mutants, indicating that the RhoGAP activities were down-regulated in T301K, S308I, and the double mutants. These data show that some mutations identified in cancer patients are biological relevant and we unexpectedly discover a regulatory site around DLC-1 aa 301 to 308 for its COOH-terminal RhoGAP domain.

Discussion

Previous mutation screens of the *DLC-1* gene in tumor DNA samples have suggested that mutations in the coding region are rare events (3, 14–17). Only one exonic missense mutation (T522A) was identified from those studies (17). In addition, eight exonic SNPs were identified, and only one of them resulted in an aa change (V354M). In contrast, our mutation analyses have been focused on the coding region for the FAT domain (aa 201–500) and have identified 8 mutations and 1 SNP (V354M) in 96 genomic and cDNA samples, demonstrating that mutations in DLC-1 are not as rare as suggested. Although the missense mutations identified in this study did not seem to affect the focal adhesion localization, two mutations compromised the tumor suppression activities of DLC-1. Our data indicate that further mutation screenings are highly warranted.

Numerous studies have described the down-regulation or absence of DLC-1 expression in a variety of cancers (2, 3). However, some of these studies also showed high frequencies of up-regulation of DLC-1 mRNA in cancers. For example, DLC-1 levels were up-regulated in 37%, 41%, and 39% of colon, stomach, and rectum cancers, respectively (18). Our identification of functional relevant mutations in DLC-1 offers a potential explanation for the up-regulation detected in cancer patients.

In this study, we have unexpectedly revealed a DLC-1 RhoGAP regulatory site away from its RhoGAP domain. Several GAPs are regulated by either protein-protein or protein-lipid interactions, and/or posttranslational modification (19). It is very likely that there is a binding site around residue 301 to 308 for a regulator that allosterically activate RhoGAP activity. When the recruitment of this regulator was abolished by mutations, RhoGAP activity was compromised. Alternatively, it is also possible that the RhoGAP activity is regulated by the phosphorylation status of this region that either leads to a conformational change or creates the binding site for the regulator. Interestingly, all missense mutations except one identified in this study show changes on aas containing a hydroxyl group, although it is currently unknown whether these residues are phosphorylated *in vivo*. Furthermore, the FAT region is serine rich with 55 (18%) serine residues within the fragment. Among them, two potential phosphorylation sites for AKT (RXRXXS/T), eight sites for PKC (S/TXR/K), and two sites for cyclic AMP-dependent protein kinase (R/KR/KX S/T) were predicted using Motif Scan.[1] At least one phospho-serine site (S329) has been formally reported (20), suggesting that the posttranslational modification may contribute to the allosterical regulation. We are currently investigating potential regulatory mechanisms involving the RhoGAP activity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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[1] http://myhits.isb-sib.ch?cgi-bin/motif_scan

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