DLC1 interaction with S100A10 mediates inhibition of in vitro cell invasion and tumorigenicity of lung cancer cells through a RhoGAP-independent mechanism

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ABSTRACT

The DLC1 gene encodes a Rho GTPase-activating protein (RhoGAP) that functions as a tumor suppressor in several common human cancers. The multidomain structure of DLC1 enables interaction with a number of other proteins. Here we report that the pro-inflammatory protein S100A10 (also known as p11), a key cell surface receptor for plasminogen which regulates pericellular proteolysis and tumor cell invasion, is a new binding partner of DLC1 in human cells. We determined that the two proteins colocalize in the cell cytoplasm and that their binding is mediated by central sequences in the central domain of DLC1 and the C-terminus of S100A10. Because the same S100A10 sequence also mediates binding to annexin 2, we found that DLC1 competed with annexin 2 for interaction with S100A10. DLC1 binding to S100A10 did not affect DLC1's RhoGAP activity, but it decreased the steady-state level of S100A10 expression in a dose-dependent manner by displacing it from annexin 2 and making it accessible to ubiquitin-dependent degradation. This process attenuated plasminogen activation and resulted in inhibition of in vitro cell migration, invasion, colony formation, and anchorage-independent growth of aggressive lung cancer cells. These results suggest that a novel GAP-independent mechanism contributes to the tumor suppressive activity of DLC1, and highlight the importance and complexity of protein-protein interactions involving DLC1 in certain cancers.
INTRODUCTION

DLC1 gene encodes a Rho GTPase-activating protein (RhoGAP) and is a potent tumor suppressor gene in several major human cancers. Loss or down-regulation of DLC1 expression, caused by genomic alterations or epigenetic modifications, frequently leads to activation of Rho GTPases, a key mediator of human oncogenesis (1-4). Transcriptional reactivation of silenced DLC1 gene in tumor cells suppresses their proliferation and migration, induces apoptosis in vitro, and inhibits in vivo tumorigenicity and development of metastases (1).

The data accumulated over the past several years underscore the complexity of DLC1 function. Initially, DLC1’s oncosuppressive effects were attributed to its RhoGAP activity, which negatively regulates several members of the Rho family of small GTPases that have a significant role in cell growth, morphogenesis, cell motility, cytokinesis, trafficking, organization of cell cytoskeleton, transformation, and metastasis (5, 6). Subsequently, RhoGAP-independent oncosuppressive mechanisms also have been identified using DLC1 GAP mutants (7-9). Given the multidomain structure of DLC1, which, in addition to the RhoGAP domain, includes an N-terminal sterile alpha motif (SAM) domain and a C-terminal steroidogenic acute regulatory protein (StAR)-related lipid-transfer (START) domain, it is not surprising that DLC1 interacts with proteins other than Rho GTPases. A yeast-two-hybrid screening identified several binding partners of DLC1 such as the members of the tensin family of focal adhesion proteins that act as a link between the actin cytoskeleton and the cytoplasmic tails of integrins (8, 10-12). Cooperation between DLC1 RhoGAP and tensin-binding activities suppresses human lung cancer cells’ migration, although the two functions are not interdependent (8).

Recently we demonstrated that the interaction of DLC1 with p120RasGAP inhibited the RhoGAP activity of DLC1 and its antiproliferative effect in human colon tumor cells (13). Others showed that binding of DLC1 SAM domain to elongation factor 1A1 (EF1A1) could mobilize EF1A1 to the membrane periphery and membrane ruffles, thus suppressing cell migration through a GAP-independent mechanism (14).

Among other binding partners of DLC1 identified by yeast-two-hybrid screening was S100A10, also known as p11 or annexin 2 light chain, a member of the S100 family of small
dimeric EF-hand type Ca\textsuperscript{2+} binding proteins (15). Here we present evidence that DLC1 interacts directly with S100A10 in human cells. Also, we localized the respective binding sites of the two proteins and determined the biological relevance of their interaction. In addition, we found that in lung cancer cells S100A10 expression is negatively regulated by the DLC1 in a dose-dependent manner. Because S100A10 belongs to a family of calcium-binding proteins that regulate the pericellular proteolysis facilitating invasive program of tumor cells, our results point to a possible new role of DLC1 protein, which, by reducing availability of S100A10, negatively affects plasminogen activation and impedes invasion of tumor cells – the steps instrumental to a metastatic process.

MATERIALS AND METHODS

Cell lines and antibodies

The human breast carcinoma (MDA-MB-231), non-small-cell lung cancer (NSCLC) (A549 and H1395), human embryonic kidney (HEK 293) and human primary small epithelial airway (PCS301-010) cells were all obtained from ATCC (Manassas, VA), cultured for less that 6 months and were not reauthenticated. HEK 293 cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum, whereas the others were cultured in RPMI-1640 medium (Invitrogen) containing 10% fetal calf serum at 37 °C in a humidified 5% CO\textsubscript{2} atmosphere. The antibodies used were obtained from the following sources: anti-annexin 2, anti-DLC1 (BD Transduction Laboratories, Franklin Lakes, NJ); anti-annexin 2 (BD Transduction Laboratories); anti-S100A10 (BD Transduction Laboratories or Abcam, Cambridge, MA); anti-GST, anti-actin, agarose-conjugated anti-ubiquitin (Santa Cruz Biotechnologies, Santa Cruz, CA); and anti-V5 (Invitrogen).

Plasmid constructions and transfection

The human annexin 2 and ubiquitin cDNA expression vectors were acquired from Origene (Origene, Rockville, MD). Full-length or truncated cDNA fragment encoding DLC1 or S100A10 with N-terminal V5 or GST tags were generated by standard PCR methods and
subcloned into the pcDNA3.1/nV5 or pDEST™27 vector (Invitrogen), respectively. DLC1 deletion mutant (Δ348-354) and GAP-dead mutant (R718E) were generated with pcDNA3.1/nV5 DLC1 as template, according to the instructions of the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), using the following primers: forward DLC1 Δ348-354, 5’-GCTTCGATCCTTTCAATCAGGAGCAGAACTTTAAGAACCG-3’, reverse DLC1Δ348-354, 5’-CGGTTCCTTTAAAGTTCTGCTCCTGATTGAAAGATCGAAGC-3’, forward DLC1 R718E, 5’-GACATGCTGAAGCAGTATTTTGAGGATCTTCCTGAGCCACTAATG-3’, reverse DLC1 R718E, 5’-CATTAGTGGCTCAGGAAGATCCTCAAAATACTGCTTCCAGCATGTC-3’. All constructs were verified by DNA sequencing. DNA transfection was carried out using Lipofectamine 2000 according to the manufacturer’s protocol.

**Transient adenovirus DLC1-mediated expression**

An adenovirus harboring the DLC1 cDNA was prepared and purified with the use of Viralbind Adenovirus Purification kit (Cell Biolab, San Diego, CA) as previously described (6, 16). The viral titer (plaque-forming units (PFU)) was determined with HEK293 cells (recommended by the manufacturer) using QuickTiter Adeno Quantitation kit (Cell Biolabs). A549 and H1395 were seeded at 1×10^6 cells/100 mm dish 24 hours before adenoviral infection. After 48 hours of infection, cells were cultured in fresh complete medium for further use.

**Co-immunoprecipitation and immunoblotting**

DLC1-positive MDA-MB-231 cells were lysed in immunoprecipitation lysis buffer (50 mM Tris-HCL, 150 mM NaCL, 5 mM EDTA, 0.5% NP-40) supplemented with proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and cleared by centrifugation at 12,000 g for 20 minutes at 4°C. A total of 500 μg of pre-cleared cell lysates were incubated with 1 μg of anti-S100A10 monoclonal antibodies (Abcam) and rotated at 4°C overnight. The immunocomplexes were recovered by incubation with 30 μL of 50% of protein G-Sepharose slurry (Zymed, Inc. San Francisco, CA) at 4°C for 4 hours. The pellets were washed four times with lysis buffer and boiled in loading buffer and subjected to immunoblotting analyses using an anti-DLC1 or anti-S100A10 antibody.
Microscopy and image acquisition

Digital photomicrographs were recorded as 24bit RGB files using a Nikon Eclipse TE2000-U inverted microscope coupled with Nikon digital camera DXM 1200F and controlled by ACT-1 (V 2.63) imaging software (Nikon, Melville, NY, USA) installed on Dell computer running Windows XP operating system. Color photographs were taken using Canon Powershot digital camera. For confocal examination cells were grown on glass chambers, incubated at 37°C for 24 hours, fixed and permeabilized in ice-cold methanol, and then blocked with 1% BSA at room temperature. Slides were stained with an anti-DLC1 (H260, Santa Cruz Biotechnology) or an anti-S100A10 monoclonal antibody (Abcam) followed by anti-rabbit Alexa 488- or anti-mouse Alexa 568-conjugated secondary antibody (Molecular Probes, Eugene, OR). Processed slides were mounted in Vectashield mounting medium containing DAPI (Vector Lab,Burlingame, CA, USA) and examined on a Zeiss LSM 510 NLO or a Zeiss LSM 510 confocal system (Carl Zeiss Inc, Thornwood, NY, USA) coupled with an Zeiss Axiovert 200M microscope. Images were collected in Zeiss AIM software (v 4.0) using a 63x Plan-Apochromat 1.4 NA or 20 x Plan-Apochromat 0.75 NA oil immersion objective and a multi-track configuration where the Alexa 488 (green fluorophore), Alexa 568 (red fluorophore) and DAPI (blue fluorophore) signals were sequentially collected in separate PMTs with a BP390-465nm, BP500-550nm and BP565-615 filters (for Zeiss LSM510 NLO), or BP385-470nm, BP505-530nm and LP560 filter (for Zeiss LSM510) after excitation with 488nm, 543 and 750 nm (for Zeiss LSM510 NLO) or 364nm, 488nm, 543nm (LSM510) laser, respectively.

In vitro pull-down assay

All peptides were synthesized with an N-terminal biotin-tag by Biomatik Corporation. Peptides were purified to > 95% by HPLC, confirmed by mass spectrometry, and dissolved in 10% DMSO (v/v) at 1 mg/ml. Amounts of 100 μg of biotin-labeled peptides were mixed with 30 μl of 50% slurry of immobilized streptavidin beads (Pierce, Rockford, IL) and rotated at 4°C for 2 hours. The beads were washed three times with 1 ml of PBS. Then 500 μg of cell lysate were added into the prepared beads and rotated at 4°C overnight. The pellets were washed three times with lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40), boiled in protein-
loading buffer, and subjected to immunoblotting analysis using an anti-S100A10 monoclonal antibody (BD Pharmingen, Franklin Lakes, NJ). For GST pull-down assay, HEK 293 cells were cotransfected with a combination of plasmids expressing GST or GST fusion proteins and DLC1 or its derivatives for 48 hours, as indicated in the figures, and treated with 10μM of proteasome inhibitor MG132 (Sigma-Aldrich) for 8 hours. Cells were lysed, and 500 μg of total cell lysate were incubated with 20 μl of glutathione agarose overnight at 4°C. Pellets were washed three times with lysis buffer, and the bound proteins were eluted in loading buffer at 100°C for 5 minutes and subjected to immunoblotting analysis with anti-V5 antibody (Invitrogen), anti-GST antibody (Santa Cruz Biotechnology), and anti-annexin 2 antibody (BD Transduction Laboratories).

**Ubiquitination assay**

HEK 293 cells were cotransfected with different combinations of plasmids expressing S100A10, annexin 2, ubiquitin, and DLC1 or its derivatives as indicated in the figures. After 48 hours of transfection, cells were lysed and cell lysates were pre-cleared. Ten micrograms of agarose-conjugated ubiquitin antibody were added to 500 μg of total cell lysate and rotated at 4°C for 4 hours. The pellets were washed three times with lysis buffer, and the bound proteins were boiled in loading buffer and subjected to immunoblotting analyses.

**Plasminogen activation assay on cells surface**

Cell surface plasminogen assay was performed as described previously (17). Briefly, cells were infected with Ad-DLC1 or Ad-LacZ for 48 hours and seeded in 24-well plates (5×10^5 cells/well) 1 day before the test. Cells were rinsed three times with PBS (pH 7.4), and media were replaced with fresh phenol red and serum-free RPMI-16409 (Invitrogen) media containing 0.5μM purified Glu-plasminogen (American Diagnostica). Conditioned media was collected and cleared by centrifugation after 6 hours. The cell-mediated plasminogen activation was determined by measuring amidolytic activity of the plasmin generated from plasminogen. Plasmin activity was monitored at 405 nm in a microplate bioassay reader after addition of
plasmin fluorogenic substrate H-D-norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide (Spectrozyme #251, American Diagnostica, Greenwich, CT) at a final concentration of 200μM.

**siRNA transfection**

Stealth siRNA for S100A10 (Invitrogen), siRNA for Annexin2 (sc-29199, Santa Cruz) and control siRNA (Invitrogen), were used for knockdown studies. The S100A10 siRNA targets region AAU GUA AAC AUC AUG GUU UCC AUG G. Cells were transfected with siRNA at 100 nM for 24 hours using Lipofectamine 2000, the medium was replaced with the fresh one, and 48 hours (for siS100A10) or 72 hours (for siAnnexin2) post transfection knockdown efficiency was established by immunoblotting analysis.

**Wound-healing, matrigel invasion, and soft agar assays**

Cells were plated in a 6-well plate at 1×10⁶ cells per well and grown overnight. The confluent cell layer was scratched with a 250 μl pipette tip (Catlog #GPS-L250, Rainin, Oakland, CA) to produce a wound gap. The region of gap was photographed immediately after scratching and 24 hours later to assess the dynamics and magnitude of wound healing. An invasion assay was carried out using a modified Boyden chamber to evaluate the capability of tumor cells to migrate through Matrigel. Briefly, 2.5×10⁴ cells were plated onto each BD BioCoat GFR Matrigel invasion chamber insert (BD Biosciences, Franklin Lakes, NJ). The medium in the upper compartment was serum-free RPMI-1640 supplemented with 0.2 μM purified Glu-plasminogen (American Diagnostica), and 5% fetus bovine serum was used as a chemoattractant in the lower compartment. After incubation for 24 hours in a humidified tissue culture incubator at 37 °C with 5% CO₂, the noninvading cells were removed from the upper surface of the membrane by scrubbing. The migrated cells were fixed with 4% PFA and stained with crystal-violet. For quantification, the average number of migrating cells was calculated by counting five random fields in the central region of membrane under a light microscope at ×100 magnification. Data represent the mean obtained from triplicate membranes. Soft agar colony
assay was carried out using the cell transformation detection kit (Cat# ECM 570, Millipore, Billerica, MA) according to the manufacture’s instruction.

RESULTS

DLC1 forms complex with S100A10

An interaction between DLC1 and S100A10 was detected by yeast two-hybrid screen (Myriad Genetics, Salt Lake City, UT) when the central part of human DLC1 (residues 340-630) was used as bait to trap the S100A10 (data not shown). To confirm the interaction between these two proteins in human cells, we carried out co-immunoprecipitation assay with extracts from a breast carcinoma cell line MDA-MB-231, which expresses high endogenous levels of both DLC1 and S100A10 proteins. Consistent with the yeast two-hybrid results, interaction between DLC1 and S100A10 proteins was detected in the cell extracts (Figure 1A).

Next, to localize the subcellular compartments in which DLC1 interacts with S100A10 proteins, double immunofluorescent staining was applied on DLC1-positive/ S100A10-positive MDA-MB-231 cells, and on S100A10-positive/DLC1-negative NSCLC A549 cells infected with Ad-DLC1. We previously emphasized that the ectopically expressed DLC1 in certain cell types can have diffuse cytoplasmatic localization, whereas in some other types it may be present at focal adhesions (1). In both A549 and MB-231 samples either endogenous or ectopic DLC1 protein (green) was present in the cytoplasm and membrane region, whereas S100A10 protein (red) was confined in cytoplasm. The two proteins colocalized (yellow) in the submembrane and cytoplasm regions (Figure 1B).

A 7-amino acid motif STFNNVV on DLC1 mediates the binding to C-terminus of S100A10

We next sought to identify the binding sites required for DLC1/S100A10 interaction. Sequence analysis revealed that two motifs within aa 340-630 of DLC1 have a pattern of hydrophobic amino acids that is similar to the region of annexin 2 that carries out binding to S100A10 (18) (Figure 2A, upper). To determine whether these motifs are responsible for binding of DLC1 to S100A10, we carried out biotin-labeled peptide pull-down assay. DLC1 peptide STFNNVV from aa 348-354 showed a strong binding to S100A10, whereas the other peptide,
WAVPKFMKRIKV from aa 619-630, failed to bind (Figure 2A, lower). To further see whether the STFNNNVV motif is required for DLC1 binding to S0100A10, a deletion mutant of DLC1 (Δ348-354) was constructed. In contrast to the full length 1091-aa DLC1, the deletion mutant Δ348-354 failed to bind to S100A10, suggesting that the seven amino acid motif is likely to be the only binding domain for S100A10 (Figure 2B). For S100A10, deletion mapping showed that C-terminus of S100A10 is required for the binding to DLC1 central part (Figure 2C, D).

**DLC1 competes with annexin 2 for binding to S100A10**

Because both DLC1 and annexin 2 bind to the same region of S100A10, we sought to determine whether there is a competition for the binding site between these two proteins. For that purpose, we used HEK 293 cells deficient in all three proteins and demonstrated that the expression of either truncated or full-length DLC1 significantly decreased binding of annexin 2 to S100A10 (Figure 3), thus confirming the competition between the two for the C-terminus site of S100A10.

**S100A10 is subjected to ubiquitination in the presence of DLC1**

S100A10 alone is rather unstable, and its physical association with annexin 2 protects S100A10 from ubiquitin-dependent proteolysis (19). To test whether competitive binding of DLC1 maintains that protection or exposes S100A10 to ubiquitin-mediated degradation, we carried out in vivo ubiquitination assay. We ectopically expressed in HEK 293 cells different combinations of ubiquitin, annexin 2, S100A10, and either truncated, full-length or deletion mutant (Δ348-354) DLC1 (Figure 4). Consistent with previous observations, S100A10 alone (lane 2) was easily ubiquitinated, whereas binding with annexin 2 blocked that process (lane 3). Such a protective effect, however, was impaired by both truncated (lane 4) and full length DLC1 (lanes 5, 6), but not by deletion mutant (Δ348-354) DLC1 (lane 7). Increased DLC1 expression (line 6) lead to a stronger ubiquitination of S100A10. Together, these data show that competitive binding of DLC1 to S100A10 removes annexin 2-mediated protection and enables subsequent ubiquitination and proteolysis of S100A10.

**DLC1 RhoGAP activity is not affected by S100A10**
To determine whether the DLC1-S100A10 interaction has an effect on DLC1 RhoGAP activity, HEK293 cells were transfected with DLC1 or S100A10 alone, and in combination. The active RhoA level was determined by a G-Lisa assay using the isolated Rho GTPase binding domain of Rhotekin, which associates preferentially with active RhoA GTPase. Expression of S100A10 did not change active RhoA level (Figure 5), and DLC1 showed a similar inhibitory effect on RhoA activity with or without S100A10. These data indicate that S100A10 has no effect on DLC1 RhoGAP activity and, consequently, on RhoA activity as well.

**Down-regulation of S100A10 by DLC1 attenuates S100A10-mediated plasminogen activation**

Because DLC1 removes annexin 2-mediated protection and exposes S100A10 to ubiquitination, we tested whether DLC1 directly affects S100A10 protein levels. Transfection of A549 cells with wild type DLC1 and the DLC1 GAP-dead mutant R718E decreased the amount of S100A10 protein, while the DLC1 mutant that did not bind to S100A10 (Δ348-354) had no effect (Figure 6A, left panel). Neither DLC1 nor DLC1 GAP-dead mutant R718E had any effect on Annexin2 protein levels suggesting that the reduction of S100A10 was not caused by down-regulation of Annexin2 (Figure 6A, right panel). Restoration of DLC1 expression in A549 cells and in the DLC1-deficient H1395 cells, which abundantly express endogenous S100A10 and annexin 2, resulted in a dose-dependent decrease in S100A10 levels (Figure 6B). S100A10 is a key plasminogen receptor and regulator of plasminogen activation (17, 20, 21). We therefore examined the effect of DLC1 on S100A10-mediated plasminogen activation. As shown in Figure 6C, ectopic expression of DLC1 transduced by adenovirus infection (100 MOI) attenuates the plasminogen activation.

**Down-regulation of S100A10 inhibits tumor cells proliferation**

Based on the results showing that DLC1/S100A10 interaction decreased S100A10 expression, we examined the effect of down-regulation of S100A10 on tumor cell proliferation. Treatment of DLC1-negative A549 cells with siRNA significantly reduced S100A10 levels (Figure 7A). Most importantly, the reduction resulted in a marked inhibition of cell migration, invasiveness, and colony formation in semisolid media, an in vitro measure of tumorigenicity, as
compared with the controls (Figure 7B-D). We then evaluated whether the introduction of DLC1 variants, namely wild-type DLC1 (GAP-competent and S-100 competent, FL), DLC1R718E (GAP-incompetent, S-100 competent) and DLC1Δ348-354 (GAP-competent, S100-incompetent) has additional repercussions on the proliferation and migration of A549 cells. Two GAP-competent DLC1 variants, i.e., wild type DLC1 and Δ348-354 DLC1, strongly inhibited cell growth (Figure 7E) and migration (supplementary Fig.1) although that effects was somewhat attenuated in the case of S-100 incompetent Δ348-354 DLC1 mutant. Interestingly, GAP-dead mutant (R718E), which is able to bind S100A10, also manifested suppressive effect on cell growth (Figure 7E) and migration (supplementary Fig.1), thus supporting the concept that a RhoGAP-independent mechanisms contributes to the oncosuppressive activity of DLC1.

**DISCUSSION**

The multiple-domain structure of DLC1 confers increased versatility in protein-protein interaction beyond binding to Rho GTPases. In this study, we identified S100A10, a key plasminogen regulatory protein highly relevant to the process of cell invasion and metastasis (15, 20, 21), as a new binding partner of DLC1 tumor suppressor protein, and characterized the nature and consequences of their interaction. The sites responsible for binding were mapped to the C-terminus of S100A10 and to a short motif located within the central region of DLC1. We found that DLC1 competes with S100A10’s ubiquitous binding partner annexin 2 and that DLC1 binding removes the protective effects of the S100A10-annexin 2 association and exposes S100A10 to subsequent ubiquitin-mediated proteolysis. The C Terminus of S100A10 contains a polyubiquitination and degradation determinant (19). Although both DLC1 and annexin 2 bind to the C-terminus of S100A10, their binding motifs are different in sequence, which might result in a different conformation of the resulting complex. When Annexin2 binds to S100A10 it could obscure an ubiquitin ligase interaction site required for the interaction between S100A10 and ubiquitin ligase and, thus, prevent ubiquitination of the former; however, the same site might remain exposed to ubiquitin ligase when S100A10 forms a complex with DLC1. It is noteworthy that the DLC1-S100A10 interaction does not compromise DLC1’s RhoGAP activity. However, it negatively affects migration, invasion and colony formation in soft agar even in the absence of
DLC1’s RhoGAP signaling, as demonstrated by experiments with the GAP-dead DLC mutant. If anything, the interaction of those two proteins somewhat intensifies DLC1’s inhibitory effects on migration, invasion and colony formation. GAP-competent/S100A10 incompetent DLC1 deletion mutant (Δ348-354) shows slightly weaker oncosuppressive effects compared to the wild-type DLC1, which could be attributed to its failure to bind S100A10 and, by reducing its availability, synergistically complement DLC1’s Rho-GAP-based suppression. These data reveal a novel GAP-independent mechanism that contributes to the role of DLC1 in suppression of early steps in the cascade of molecular events leading to metastasis.

The DLC1-S100A10 interaction is mediated through the central part of DLC1. Although this region that links the SAM and RhoGAP domain is largely unstructured, the serine-rich sequence contains multiple putative phosphorylation sites, suggesting that it plays a regulatory role in DLC1 function (1, 22). For example, the focal adhesion targeting (FAT) unit (aa 265–459 in DLC1)(23), which anchors DLC1 at focal adhesion sites by cooperating with members of the tensin family (8, 11) via the motif SIYDNV (aa 440–445 in DLC1) and/or a recently found PTB binding sequence (24), is located within this region (8) just downstream of the S100A10 binding site (aa 348-354 in DLC1). In addition, the phosphorylation-dependent interaction with 14-3-3 adaptor proteins is mapped within the same region and is shown to negatively regulate DLC1 RhoGAP activity (25). Interestingly, mutations within this segment (residue 301-308), which might be a potential binding site for a regulator that allosterically activates RhoGAP activity, had no effect on the subcellular localization of DLC1 but reduced RhoGAP activities (22). Although the other two members of the DLC family of genes, DLC2 and DLC3, are very similar to DLC1 in terms of domain organization, the motif STFNNV is not conserved among them, strongly suggesting that they may not be able to bind S100A10 and that this capacity is unique to DLC1.

Our results suggest that DLC1-S100A10 interaction contributes to the DLC1 tumor-suppressive activity in a GAP-independent manner, which is consistent with several reports showing that DLC1 exerts its functions not only through its RhoGAP activity (7). Indeed, both the focal adhesion localization and RhoGAP activity of DLC1 are required for its tumor-suppressive activity (8, 9, 22). Interaction of EF1A1/DLC1 is also consequential to the oncogenic process through a GAP-independent mechanism (14). Moreover, our data with the
inhibitory effect of DLC1 GAP-deficient mutant (R718E) on anchorage-independent growth of NSCLC DLC1-deficient cells in soft agar as a measure of tumorigenicity in vitro show the complexity of tumor suppression process.

S100A10 belongs to the S100 family of proteins implicated in various intracellular processes such as cell growth, cell cycle, transcription, and differentiation (15). It is over-expressed in various cancers and plays a role in regulation of cellular invasiveness by stimulating plasminogen activation (20, 21, 26, 27). Our observation that reduced availability of S100A10 negatively affects cell growth in vitro is consistent with a previous observation that S100A10 is mainly expressed in regions with strong proliferating capacity (28) and suggests a role for S100A10 in the regulation of cell proliferation. However, mechanism(s) by which S100A10 influences cell growth remain to be determined. Previous studies have demonstrated that several of the S100 family proteins, such as S100A1, S100A8/9, and S100P, could promote cell growth by binding to their ligand RAGE (receptor for activated glycation end-products), thus triggering a RAGE-dependent pathway (29-31). Because S100A10 cannot bind to RAGE, the mechanism of its eventual influence over cell proliferation is probably quite different. Alternatively, S100A10 may bind to BAD protein, negatively affecting BAD-induced apoptosis (32).

The inhibitory effect of DLC1 on plasminogen activation likely results from DLC1’s ability to reduce S100A10 availability by both down-regulating its expression and aiding its ubiquitin-mediated proteolysis. S100A10 plays a key role in regulation of plasminogen activation and enhances plasminogen activator-dependent conversion of plasminogen to plasmin, thus promoting tumor invasion and metastasis. Loss of S100A10 results in a marked decrease in plasmin generation as well as in invasion capacity (15, 20, 21). It has been well-established that the role of wild type S100A10 in plasminogen activation depends largely on its two C-terminal lysine residues (15). S100A10-annexin 2 complex is twofold more efficient in stimulating activation of plasminogen than S100A10 alone, and 23-fold more efficient than annexin 2 alone (17). By competing with annexin 2, DLC1 may disrupt the S100A10-annexin 2 complex and allosterically alter the plasminogen-binding capability of S100A10 by occupying the two lysine residues to which plasminogen generally binds (15, 17). Further studies certainly are required to resolve this issue.
Collectively, our results provide new evidence supporting DLC1 also has onco-suppressive activity through a GAP-independent manner, highlighting the importance and complexity of protein-protein interactions involving DLC1 in a subset of cancers.

REFERENCES


**LEGENDS OF FIGURES**

**Figure 1**: DLC1 forms a complex with S100A10. (A) Extracts from human breast cancer cell MDA-MB-231 were immunoprecipitated (IP) using DLC1 (left panel, lane 1) or S100A10 antibody (right panel, lane 1) and analyzed by immunoblotting. Normal rabbit or mouse serum (lane 2 in both panels) was used as IP control. Cell lysate (lane 3 in both panels) was used as a positive control. (B). Confocal immunofluorescent colocalization of DLC1 and S100A10 in (i) S100A10-positive/DLC1-positive human breast cancer MDA-MB-231 cells. (ii) S100A10-positive/DLC1-negative human NSCLC A549 cells infected with AdDLC1 or (iii) transfected with LacZ, and (iii) in S100-negative/DLC1-negative HEK293 cells used as a negative control. Scale bar 20μm.

**Figure 2**: A 7-amino acid motif on DLC1 central region carries out the binding with C-terminus of S100A10. (A) Sequence comparison of S100A10 binding motif between Annexin2 and DLC1. The highlighted letters indicate the identical and homologue residues (top). A
7-amino acid motif STFNNVV on DLC1 central region binds to S100A10. Biotin-labeled peptides were conjugated with streptavidin beads and incubated with A549 extracts; bound proteins were analyzed by immunoblotting with anti-S100A10 antibody (bottom). (B) GST pull down assay and WB analysis of extracts from HEK 293 cells cotransfected with a V5-tagged full length DLC1 or a deletion mutant (Δ348-354) DLC1, and a GST-tagged full length S100A10. (C). Schematic representation of GST-tagged S100A10 fragments and V5-tagged DLC1 fragment (top). Full-length S100A10 (1-97) or truncated S100A10 (1-79) with GST tag was cotransfected with DLC1 fragment (340-630) with V5 tag in HEK 293 cells; GST pull-down assay was performed using transfected cell extracts and then subjected to WB analysis (bottom). (D) Schematic representation the DLC1-S100A10 interaction. The numbers refer to the amino acids for the respective domains of each protein.

Figure 3: DLC1 competes with annexin 2 for the binding with S100A10. GST-S100A10 and full-length DLC1 or truncated DLC1 with annexin 2 were cotransfected in HEK 293 cells at the indicated ratio. Forty-eight hours after transfection cells were treated with 10 μM of proteinase inhibitor MG132 for additional 8 hours, harvested, and 500 μg of cell lysate was subjected to GST pull-down assay. The annexin 2 signal was reduced whenever the DLC1 was used as a competitor.

Figure 4: Effect of DLC1 on S100A10 ubiquitination. HEK293 cells were transfected with different combinations of ubiquitin, S100A10, annexin 2, full-length DLC1, truncated (340-630) DLC1, and deletion mutant (Δ348-354) DLC1, as indicated in the figure. Forty-eight hours after transfection, cells were lysed and equal amounts of cell lysates were subjected to immunoprecipitation with agarose-conjugated ubiquitin antibody. Ubiquitinated S100A10 in immunoprecipitates was detected by immunoblotting with S100A10 antibody (left). The expression of each transfected proteins is shown on the right.

Figure 5: Effect of S100A10 on RhoA activity. HEK 293 cells were transfected with plasmids expressing either S100A10 or DLC1, using Lipofectamine 2000 as described. Forty-eight
hours after transfection, cells were lysed and equal amounts of cell lysates were subjected
to G-Lisa RhoA assay.

**Figure 6:** DLC1 down-regulates S100A10 in a dose-dependent manner and attenuates
S100A10-mediated plasminogen activation. (A) Whole cell lysates from A549 cells transiently transfected with V5-tagged either a full length DLC1, deletion mutant (Δ348-354) DLC1, or GAP-dead mutant (R718E) DLC1, were immunoblotted with anti-V5, anti-Annexin2 and anti–S100A10 antibodies. (left panel). Also, A549 cells were treated with 100nM Annexin2 siRNA for 72 hours, and whole cell extracts were immunoblotted with anti-Annexin2 and anti-S100A10 antibodies (right panel). In both panels actin was used as loading control. (B) DLC1-negative NSCLC lines A549 and H1395 were infected with Ad-DLC1 at various multiplicities of infection (MOI) and whole cell extracts were immunoblotted with anti-DLC1 and anti–S100A10 antibodies. Actin was used as a loading control. (C) NSCLC lines A549 and H1395 were infected with Ad-DLC1 at 100 MOI for 48 hours. One day before the test, 5×10^5 cells were seeded in 24-well plates, Glu-plasminogen was added when cells reached confluency, and cell-generated plasmin activity was measured.

**Figure 7.** Down-regulated S100A10 contributes to oncosuppressive activity. (A) A549 cells were treated with S100A10 siRNA for 48 hours, and the knock-down efficiency was confirmed by immunoblotting analysis. Actin was used as a loading control. (B) Effect of S100A10 siRNA on cell migration. Scale bar 50μm. (C) Effect of S100A10 siRNA on colony formation. (D) Effect of S100A10 siRNA on cell invasiveness (Matrigel Invasion Assay), Data presented are the mean ± SD of the number of cells counted in five randomly selected fields. One of three independent experiments is shown. Statistical significance was analyzed by the Student’s t-test. Scale bar 20μm. (E) A549 cells were transfected with vectors for either wild type DLC1, the GAP-dead mutant DLC1 (R718E) or deletion mutant (Δ348-354) DLC1. After 48 hours the cells were seeded in 6-well plates for colony formation in soft agar (0.4%) with selective medium containing 600μg/ml of G418 and photographed after 3 weeks. Data shown represent the mean ± SD
of triplicate wells of colonies and are representative of three independent experiments. Statistical significance was analyzed by the Student’s t-test. Scale bar 50μm.
Figure 1

A

MDA-MB 231

IP: DLC1
WB: S100A10

1 2 3

DLC1
S100A10

B

S100A10  DLC1  DAPI  Merged

1 2 3 4

20 μm 20 μm 20 μm 20 μm

i ii iii iv
A

Anexin 2 binding motif:
DLC1 (aa 348-354):
DLC1 (aa 619-630):
Control peptide:

Pull down: Avidin

B

Vector Δ348-354 DLC1 DLC1
Pull down GST IB: V5

DLC1
IB: V5
IB: GST

S100A10

C

S100A10 fragments DLC1 fragment
1 67
1 79

Pull down: GST

D

Focal Adhesion Targeting Region

DLC1

S100A10

EF1 EF2 Plectinogen Binding
Figure 2

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<th>GST-S100A10</th>
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</tr>
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DLC1 interaction with S100A10 mediates inhibition of in vitro cell invasion and tumorigenicity of lung cancer cells through a RhoGAP-independent mechanism

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