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Direct Association of TSLC1 and DAL-1, Two Distinct Tumor Suppressor Proteins in Lung Cancer[1]

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Abstract

The tumor suppressor gene *TSLC1*, which we recently identified in human non-small cell lung cancer, encodes a membrane glycoprotein of the immunoglobulin superfamily. Here, we report that TSLC1 directly associates with DAL-1, a gene product of another lung tumor suppressor belonging to the protein 4.1 family. TSLC1 additionally interacts with the actin filament through DAL-1 at the cell-cell attached site where the complex formation of TSLC1 and DAL-1 is dependent on the integrity of actin cytoskeleton. Redistribution of both TSLC1 and DAL-1 to the newly generated membrane ruffling areas suggests that these proteins are also involved in cell motility accompanying the actin rearrangement. Furthermore, restoration of TSLC1 expression strongly suppressed the metastasis of a human non-small cell lung cancer cell line, A549, from the spleen to the liver in nude mice. These findings, together with frequent loss of their expression in lung cancers, suggest that TSLC1 and DAL-1 play a critical role in the same pathway involved in the suppression of lung tumor formation and metastasis.

Introduction

TSLC1 is a tumor suppressor gene in NSCLC[3] that was identified by functional complementation through the suppression of tumor formation by the human lung adenocarcinoma cell line A549 (1). Two-hit inactivation by loss of one allele and promoter methylation or inactivating mutations in the remaining allele are observed in ~40% of primary NSCLC tumors as well as in 20–30% of tumors from the liver, pancreas, and prostate (1, 2). An extracellular domain of TSLC1 contains three immunoglobulin-like C2-type loops, suggesting the involvement of cell adhesion. A truncating mutation of *TSLC1* corresponding to the cytoplasmic domain in a primary NSCLC suggests that this short domain of 47 amino acids is also important for tumor suppression. This cytoplasmic domain of TSLC1 contains a protein 4.1 binding motif and shows significant homology with that of human glycophorin C and *Drosophila* Neurexin IV. Glycophorin C associates with protein 4.1 and plays crucial roles in membrane deformity of erythrocytes (3), whereas Neurexin IV binds to Coracle, a *Drosophila* protein 4.1 homologue, and participates in the maintenance of the septate junction (4). The protein 4.1 family molecules present significant similarity with ezrin, radixin, and moesin (ERM subfamily) and merlin, a gene product of the tumor suppressor *NF2*, all of which are known to act as anchors for several transmembrane proteins to the actin cytoskeleton through their protein4.1/ezrin/radixin/moesin domain and the spectrin-actin binding domain (5, 6). Therefore, it is reasonable to speculate that the activity of tumor suppression by TSLC1 would also require its anchoring to plasma membrane through a molecular apparatus similar to that seen in glycophorin C or Neurexin IV. The protein 4.1 family consists of four distinct molecules, including 4.1R, 4.1G, 4.1N, and DAL-1 (4.1B; Ref. 5). Although the former three molecules are not reported to be involved in lung carcinogenesis, DAL-1 was shown to be down-regulated in considerable portions of primary lung tumors as well as lung cancer cell lines (7). Furthermore, restoration of DAL-1 expression to a normal level in NSCLC cell lines significantly suppressed cell growth *in vitro*, which suggests its role as a tumor suppressor (7). These reports led us to examine the possible interaction of TSLC1 and DAL-1, two distinct tumor suppressor proteins in human lung cancer.

Materials and Methods

Cell Lines. A549, PC-14, and Cos-7 cells were obtained from the Riken cell bank (Tsukuba, Japan); ABC-1, RERF-LC-MS, RERF-LC-OK, VMRC-LCD, and A431 cells were from the Health Science Research Resources Bank (Osaka, Japan); and U251, Calu-3, NCI-H441, NCI-H522, SK-LU-1, NCI-H596, and a human embryonic kidney cell line (HEK-293) were from the American Type Culture Collection (Manassas, VA). Cells were cultured according to the supplier's recommendations.

Expression Vectors, Antibodies, and Chemicals. DAL-1 cDNA was provided from Dr. Takahiro Nagase (Kazusa DNA Research Institute) and cloned into a pcDNA3.1/V5-His TOPO TA vector to generate pcDNA3.1/V5-DAL-1. DAL-1-65 cDNA was amplified by reverse transcription-PCR using the human adult lung poly(A) RNA with the primers 5′-TAGCAGTAAACTCTCTCG-GTTTTCA-3′ and 5′-TCCACTGGACTCCGTCTTGGTTTCC-3′ and subcloned into a pcDNA3.1/V5-His TOPO TA vector. For the construction of the expression vectors of GST-TSLC1 fusion proteins, three fragments corresponding to the COOH-terminal portions of TSLC1 were amplified by PCR from pcTSLC1 (1) and subcloned into pGEX-4T-1 (Amersham Pharmacia). A synthetic polypeptide of 18 amino acids, N-INAEGGQNNSEEKKEYFI-C, at the carboxyl end of TSLC1 fused with KLH was used as an immunogen to raise rabbit anti-TSLC1 pAb CC2.

Immunoprecipitation and Western Blotting. Twenty-four h after transfection with pcDNA3.1/V5-DAL-1, HEK293 cells were washed and treated with a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (Calbiochem)] on ice for 10 min and centrifuged, and protein content was determined using a protein assay reagent (Bio-Rad). One μg of the antibody was added to 500 μg of the cell lysate and incubated overnight at 4°C, then a 50% suspension of Protein A-Sepharose 6MB (Amersham Pharmacia) was added and incubated for 1 h at room temperature. Beads were then washed four times with a lysis buffer and resuspended in a NuPAGE sample buffer (Invitrogen). Immunoprecipitates or

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; pAb, polyclonal antibody; mAb, monoclonal antibody; GST; glutathione *S*-transferase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

5 μg of whole cell lysates were electrophoresed on 4–12% NuPAGE minigels (Invitrogen) and transferred to a polyvinylene difluoride membrane (Millipore) with a semi-dry transfer blot system (Bio-Rad). After blocking with Tris-buffered saline containing 1% Tween 20 and 5% skim milk for 30 min, the filters were incubated with primary antibody for 1 h, washed, and incubated with the appropriate horseradish peroxidase-labeled secondary antibody (Amersham Pharmacia). Specific proteins were detected using an enhanced chemiluminescence system (Lumi-Light^{PLUS}; Roche).

GST Pull-Down Assay. GST-TSLC1 fusion proteins expressed in *Escherichia coli* were purified using glutathione Sepharose 4B (Amersham Pharmacia). [^{35}S]Methionine-labeled DAL-1-65 was synthesized in reticulocytes from pcDNA3.1/V5-DAL-1-65 using a TNT T7 Quick Coupled transcription/translation system (Promega). For *in vitro* binding, labeled proteins were incubated with equal amounts of GST-TSLC1 fusion proteins coupled with glutathione Sepharose beads for 1 h at 30°C in a NETN buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.5% NP40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin]. After incubation, the beads were washed with the NETN buffer and subjected to SDS-PAGE (10–20% ready gel; Bio-Rad). The gels were stained with Coomassie Brilliant Blue (ICN), and binding of ^{35}S-labeled proteins was detected by autoradiography.

Immunofluorescence Microscopy Analysis. HEK293 and U251 cells transfected with pcDNA3.1/V5-DAL-1-65 were selected against G418 for 2 weeks, and pooled clones were obtained. Cells were seeded on a Collagen I-coated culture slide (Biocoat; Becton Dickinson) and treated with 100 ng/ml of TPA (Sigma) at 30 min or 2 μM of cytochalasin D (Sigma) at 10 h. After being washed with PBS, cells were fixed with 4% paraformaldehyde for 15 min and then with 0.2% Triton X-100 in PBS for 5 min. Cells were subsequently incubated with a blocking solution (5% normal donkey serum, 0.02% sodium azide in PBS) for 1 h and incubated overnight with primary antibodies at 4°C. The cells were washed and incubated with FITC- or tetramethylrhodamine isothiocyanate-labeled secondary antibodies (Jackson Immunoresearch) in the presence of Alexa Fluor 633 dye-labeled phalloidin (Molecular Probes) for 1 h at room temperature. Samples were then washed and mounted in 80% glycerol and viewed with a laser-scanning confocal system (Radience 2000; Bio-Rad).

Northern Blot Analysis. Poly(A) RNA was extracted from NSCLC cell lines using the FastTrack 2.0 kit (Invitrogen). One μg of poly(A) RNA was subjected in electrophoresis on 1% agarose-formaldehyde gels and transferred to Hybond-N+ (Amersham Pharmacia). A *Hin*dIII and *Xho*I fragment of 1651 bp from pcDNA3.1/V5-DAL-1-65 and a 961-bp PCR-derived fragment of TSLC1 cDNA (1) were used as probes for the detection of DAL-1 and TSLC1, respectively, after labeling with [^{32}P]dCTP by a Multiprime DNA labeling system (Amersham Pharmacia).

Examination for Metastasis from the Spleen to the Liver. Three independent A549 cell lines stably expressed full-length TSLC1 (ATSLC1, ATSLC2, and ATSLC3), and the A549 cells carrying the control plasmid pcDNA3.1-Hygro(+) were obtained as described previously (1). The metastatic activity of the A549 cell and its derivatives *in vivo* were examined as reported previously (8). In brief, 5×10^5 cells on 0.05 ml of PBS were injected into the medial splenic tip of the anesthetized BALB/c nu/nu mice (Japan Crea Laboratory, Tokyo, Japan). Eight weeks after injection, the animals were sacrificed, and all of the visceral organs were examined for macroscopic metastasis as well as micrometastasis after fixation.

Results and Discussion

The cytoplasmic domain of TSLC1 harbors a sequence of 10 amino acids that exactly matches the protein 4.1 binding motif. As shown in Fig. 1*a*, Neurexin IV, paranodin, syndecan-2, and glycophorin C also share this motif, and these proteins are shown to be directly associated with a member of protein 4.1 family molecules. This raised the possibility that TSLC1 might interact with DAL-1, a member of protein 4.1 family molecules. To analyze the possible association, we

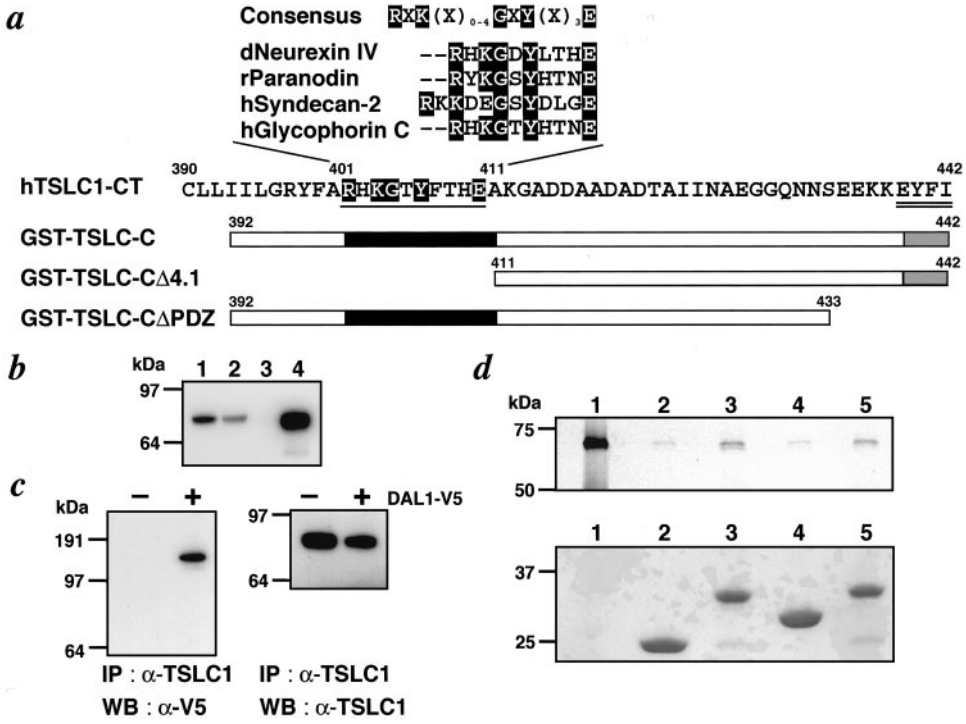

Fig. 1. Association of TSLC1 with DAL-1 *in vivo* and *in vitro*. *a*, amino acid sequence of the cytoplasmic domain as well as a portion of the transmembrane domain of TSLC1 (hTSLC1-CT). Conserved amino acid residues in the cytoplasmic domain among TSLC1 and four transmembrane proteins known to bind with proteins 4.1 are shown above the sequence of hTSLC1-CT. Consensus sequence is indicated at the *top*. *Black shadings* indicate conserved residues among the five proteins (5). h, d, and r indicate human, *Drosophila*, and rat, respectively. Note that the binding of syndecan-2 with proteins 4.1 has not been demonstrated yet. The GST-fusion protein with an entire cytoplasmic domain of TSLC1 (GST-TSLC1-C) and a couple of its deletion mutants are schematically represented below the sequence of hTSLC1-CT. *b*, detection of the TSLC1 proteins in four cell lines by immunoblotting with anti-TSLC1 pAb, CC2. *Lane 1*, HEK293; *Lane 2*, U251; *Lane 3*, Cos-7; and *Lane 4*, Cos-7 transfected with pcTSLC1. Transfection was carried out using LipofectAMINE (Invitrogen). *c*, coprecipitation of TSLC1 with DAL-1. 293 cells were transfected with pcDNA 3.1/V5-DAL-1, and the cells were immunoprecipitated with anti-TSLC1 pAb and detected by Western blotting with anti-V5 mAb (*left*; Invitrogen) or anti-TSLC1 pAb (*right*). *d*, (*top*) GST pull-down assay for the binding of [^{35}S]methionine-labeled DAL-1-65 with a series of GST-TSLC1 fusion proteins. *Lane 1*, input; *Lane* 2, GST; *Lane 3*, GST-TSLC-C; *Lane 4*, GST-TSLC-CΔ4.1; and *Lane 5*, GST-TSLC-CDPΔZ. *Bottom*, detection of a series of GST fusion proteins by staining the gel with Coomassie Brilliant Blue.

first generated a pAb, CC2, against the cytoplasmic tail of TSLC1, which detected a single molecule of exogenously expressed TSLC1 of M_r ~75,000 in Cos-7 cells. A significant amount of endogenous TSLC1 of the same size has also been observed in a human embryonic kidney cell line, HEK293, and a glioblastoma cell line, U251 (Fig. 1*b*). When DAL-1 tagged with V5 was expressed in HEK293 cells and extracts of the cells were immunoprecipitated with anti-TSLC1 pAb, DAL-1 of M_r 110,000 was coprecipitated with endogenous TSLC1 and was detected by anti-V5 mAb (Fig. 1*c*). These findings suggest that TSLC1 directly associates with DAL-1 *in vivo*. Immunoprecipitation with anti-V5 mAb could not detect coprecipitated TSLC1 in the same cell lysates, probably because exogenously expressed DAL-1 was much more abundant than endogenous TSLC1.

To localize the precise region necessary for the association of TSLC1 with DAL-1, a fragment corresponding to an entire cytoplasmic domain and a portion of a transmembrane domain of TSLC1 (TSLC-C), as well as a couple of deletion mutants that lack a possible 4.1 binding motif (TSLC1-CΔ4.1) and a PDZ binding motif (TSLC1-CΔPDZ), respectively, were constructed *in vitro* as fusion proteins with GST (Fig. 1*a*). A GST pull-down assay was then carried out with [^{35}S] methionine-labeled DAL-1-65, a central portion of DAL-1 containing an entire protein 4.1/ezrin/radixin/moesin domain, and a putative spectrin-actin binding domain. GST-TSLC1-C and GST-TSLC1-CΔPDZ significantly bound to DAL-1 almost at an equal level, whereas the binding activity of GST-TSLC1-CΔ4.1 was reduced to the same level as that of GST alone, suggesting that TSLC1 binds to DAL-1 through its protein 4.1 binding motif (Fig. 1*d*).

Next, subcellular distribution of TSLC1 and DAL-1 was examined using immunofluorescence microscopy. HEK293 cells expressing DAL-1-65 tagged with V5 were stained with anti-TSLC1 pAb for TSLC1 (red), anti-V5 mAb for V5 (green), and phalloidin for actin (magenta). TSLC1 and DAL-1 colocalized along the cell membrane, displaying honeycomb-like patterns of staining when cells are cultured in confluence (Fig. 2, *a–c*). Actin filaments were also observed at the cell periphery in a similar pattern (Fig. 2*d*). When an early stage of cell adhesion was examined by culturing cells in low density, both TSLC1 and DAL-1 were localized primarily at the cell-cell attached sites (Fig. 2, *f–h*), whereas the actin cytoskeleton was organized along an entire plasma membrane, including the cell-cell attached sites (Fig. 2*i*). Taken together, these results suggest that TSLC1 interacts with the actin cytoskeleton through DAL-1.

To additionally support this hypothesis, the actin cytoskeleton of these cells was disrupted by treatment with cytochalasin D, an inhibitor of actin polymerization. As shown in Fig. 2, *n* and *o*, cell-cell attachment was partly destroyed, and the cytoskeletal organization of actin was lost from the detached membrane (indicated by arrow). Corresponding to this change, both TSLC1 and DAL-1 also disappeared from the unattached membranes. On the other hand, TSLC1 remained associated with DAL-1 at the sites where cell-cell attachment was still intact (Fig. 2, *k–m*). These findings suggest that the colocalization of TSLC1 and DAL-1 is dependent on the integrity of the actin cytoskeleton.

To additionally investigate the role of TSLC1 and DAL1 in cytoskeletal organization, U251 cells expressing exogenous DAL-1 were treated with a phorbor ester, TPA, a potent activator of protein kinase C. TSLC1 and DAL-1 were colocalized at the cell periphery

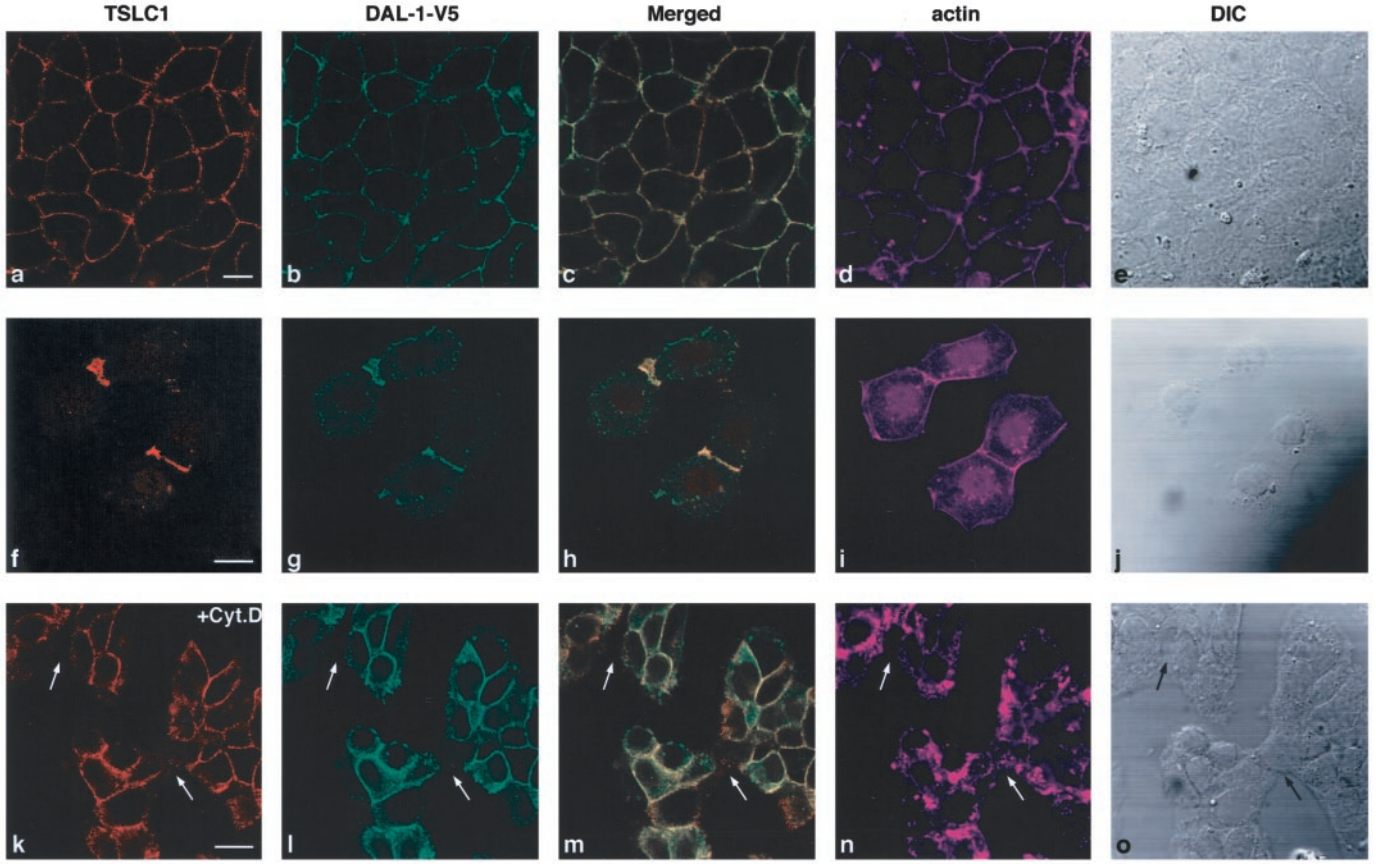

Fig. 2. Subcellular colocalization of TSLC1 and DAL-1. DAL-1-65-V5-expressing HEK293 cells were cultured in a confluent stage (*a–e*) or in low density (*f–j*), triple stained with anti-TSLC1 pAb (*a* and *f*, *red*), anti-V5 mAb (*b* and *g*, *green*), and Alexa Fluor 633 dye-labeled phalloidin (Molecular Probes) (*d* and *i*, *magenta*) and analyzed with confocal microscopy. Merged images of *red* and *green* (*c* and *h*) and images of differential interference contrast (DIC; *e* and *j*) are also shown. (*k–o*) The same cells were pretreated with 2 μM cytochalasin D for 10 h before fixation and analyzed as above. Bar, 20 μm.

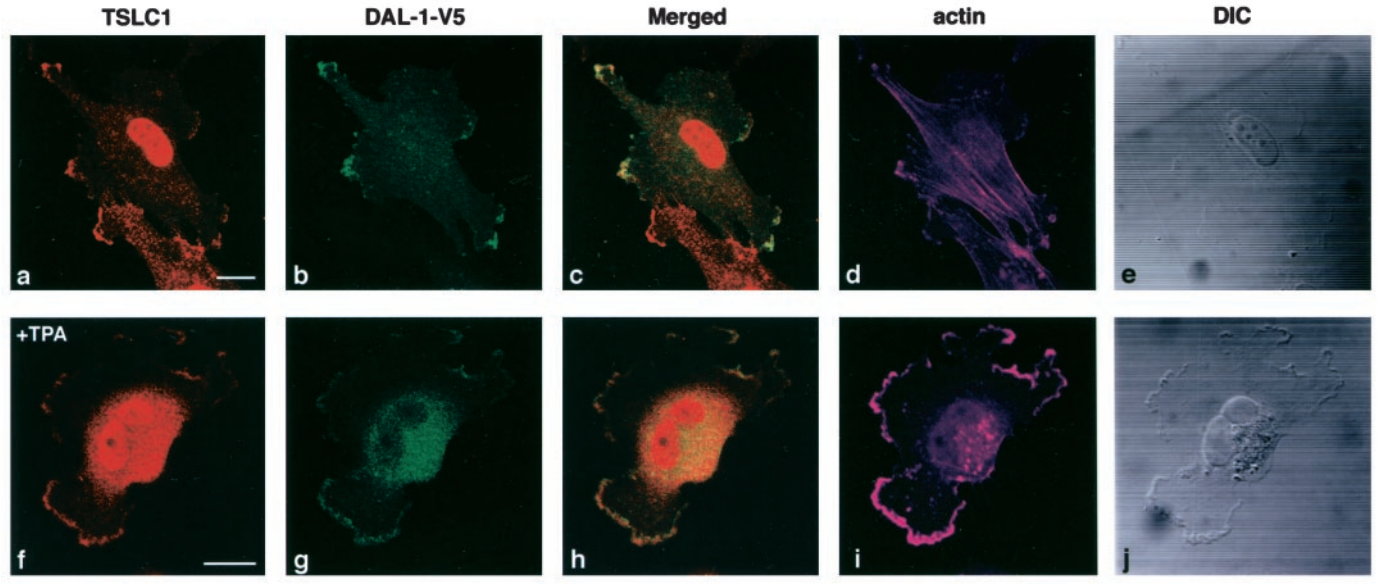

Fig. 3. Redistribution of TSLC1 and DAL-1 to membrane ruffling areas induced by TPA. U251 cells expressing DAL-1-65-V5 were treated with 100 ng/ml TPA for 0 min (*a–e*) or 30 min (*f–j*), triple stained with anti-TSLC1 pAb (*a* and *f*, *red*), anti-V5 mAb (*b* and *g*, *green*), and Alexa Fluor 633 dye-labeled phalloidin (*d* and *i*, *magenta*) and analyzed with confocal microscopy. Merged images of *red* and *green* (*c* and *h*) and differential interference contrast (DIC) images (*e* and *j*) are shown. Bar, 20 μm.

when U251 cells were cultured in low density without TPA (Fig. 3, *a–c*). Signals of TSLC1 were also detected in the nucleus, although their significance was unclear. Stress fibers of actin filaments were typically organized in U251 cells as seen in Fig. 3*d*. When cells were treated with TPA, stress fibers were broken down, but membrane ruffling was induced by reorganization of the actin cytoskeleton (Fig. 3*i*) where TSLC1 and DAL-1 were redistributed (Fig. 3, *f–h*). These results suggest that dynamic distribution of TSLC1 and DAL-1 to the actin cytoskeleton might be involved in not only cell adhesion but also cell motility. Staining of TSLC1 and DAL-1 was also detected at the perinuclear space with actin filaments, suggesting that the complex might be organized in the Golgi apparatus.

We have previously reported that *TSLC1* expression is lost or reduced in 6 of 12 NSCLC cell lines where the reduction is correlated with their *in vivo* activity of tumorigenicity and/or metastasis (1). Because down-regulation of DAL-1 expression was also reported in >50% of primary lung cancers as well as 80% of NSCLC cell lines (7), we compared the expression of *DAL-1* with that of *TSLC1* in 12 NSCLC cell lines. Northern blot analysis revealed that *DAL-1* expression was lost or markedly reduced not only in the 6 cell lines lacking *TSLC1* expression but also in 3 additional cell lines, NCI-H441, NCI-H522, and RERF-LC-OK (Fig. 4, *a* and *b*). Three cell lines, ABC-1, RERF-LC-MS, and VMRC-LCD, expressed both *TSLC1* and *DAL-1*. This finding is significant because Hirai *et al.* (8) had reported that these cells showed reduced metastatic ability from the spleen to the liver in nude mice. Thus, to examine whether TSLC1 could be directly involved in the suppression of metastasis in NSCLC, three independent A549 derivatives, ATSLC1, ATSLC2, and ATSLC3, which stably expressed full-length *TSLC1*, as well as the A549 cells transfected with only plasmid DNA, were injected into the spleen in nude mice. Twelve of 16 mice injected with A549 cells carrying the control plasmid formed tumors in the liver, as determined when the mice were sacrificed 8 weeks after injection (Fig. 4*c*). In contrast, none of the 23 mice injected with ATSLC1, ATSLC2, or ATSLC3 cells developed tumors in the liver. No tumors were detected in the original sites of injection in the spleen or other visceral organs in either group of mice. These results indicate that TSLC1 has a strong suppressor activity of metastasis *in vivo* when expressed in TSLC1-deficient NSCLC cells. Taken together, loss of TSLC1 and DAL-1 function could be involved synergistically in lung cancer formation and/or metastasis.

Lung cancer is one of the most common human cancers in the world. We demonstrated that TSLC1 and DAL-1, originally identified as lung tumor suppressors by different approaches, could be associ-

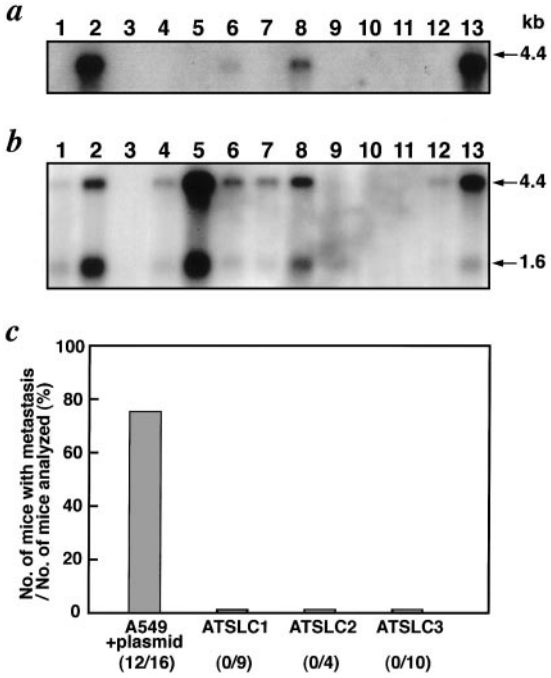

Fig. 4. Northern blot analyses of the *DAL-1* and the *TSLC1* genes in NSCLC cell lines and suppression of metastasis by TSLC1. Expression of the *DAL-1* (*a*) and the *TSLC1* (*b*) was examined in 12 NSCLC cell lines. *Lane 1*, A549; *Lane 2*, ABC-1; *Lane 3*, Calu-3; *Lane 4*, NCI-H441; *Lane 5*, NCI-H522; *Lane 6*, RERF-LC-MS; *Lane 7*, RERF-LC-OK; *Lane 8*, VMRC-LCD; *Lane 9*, PC-14; *Lane 10*, SK-LU-1; *Lane 11*, NCI-H596; *Lane 12*, A431; and *Lane 13*, normal lung. *c*, TSLC1 suppresses the metastasis of A549 cells from the spleen to the liver. The incidence of the metastasis of A549 transfected with the control plasmid and A549 transfected with TSLC1 (ATSLC1, ATSLC2, and ATSLC3) was shown by the number of mice with metastasis out of the number of mice analyzed.

ated, indicating that these two proteins act in the same cascade in the suppression of lung cancer. It is plausible that TSLC1 interacts with the actin filament through an anchoring protein, DAL-1, and participates in organizing the actin cytoskeleton and constructing stable adhesion between adjacent cells. Then, loss of either protein in cancer cells could affect normal cell adhesion and lead to invasion or metastasis to adjacent or distal tissues. Furthermore, we demonstrated that the complex of TSLC1 and DAL-1 was accumulated in TPA-induced membrane ruffling areas where the actin cytoskeleton was dynamically reorganized. This suggests that TSLC1 and DAL-1 may be involved in cell motility by transmitting the signals of cell-cell or cell-substrate interaction to the actin cytoskeleton. It has been reported that most of the actin rearrangement in cell motility is orchestrated by the Rho family of small GTPases (9) and that the induction of membrane ruffling by TPA is regulated by the coordination of Rac and Rho (10). Thus, the association of TSLC1 with DAL-1 and its dynamic distribution could be regulated by Rho GTPases.

E-cadherin, another tumor suppressor of the transmembrane adhesion molecule, also interacts with the cytoskeleton and is involved in the invasion or metastasis of gastric and several other cancers. Numbers of cytoplasmic proteins, including α- and β-catenins and adenomatous polyposis coli, associate with E-cadherin directly or indirectly and provide targets for mutation in human cancer (11). Similarly, TSLC1 and DAL-1, acting in the same cascade, could be the targets for inactivation in human lung carcinogenesis. In fact, loss of expression of *TSLC1* and *DAL-1* frequently occurs in primary lung cancer and cell lines. Our findings, however, do not exclude the possibility that TSLC1 might also be associated with other members of protein 4.1 molecules. Additional studies would elucidate the possible interaction between TSLC1 and these molecules in various tissues. TSLC1 also harbors a PDZ binding motif at the cytoplasmic end in addition to the protein 4.1 binding motif, suggesting that it can associate with additional proteins. Finding more partners of TSLC1, as well as searching for their alterations in lung cancer, would provide a deeper background for understanding the significance of this pathway in lung tumor suppression.

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