The 4.1/ezrin/radixin/moesin domain of the DAL-1/Protein 4.1B tumour suppressor interacts with 14-3-3 proteins

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The Protein 4.1 family contains at least two members that function as tumour suppressors, the neurofibromatosis 2 gene product merlin and the recently identified differentially expressed in adenocarcinoma of the lung (DAL-1)/Protein 4.1B molecule. DAL-1/Protein 4.1B loss is observed in a variety of tumours, including breast and lung cancers as well as meningiomas. We have previously demonstrated that DAL-1/Protein 4.1B interacts with some but not all merlin-binding proteins, raising the possibility that DAL-1/Protein 4.1B associates with additional unique proteins specific to its function as a negative growth regulator. Using yeast two-hybrid interaction cloning, we identified three 14-3-3 isoforms, β, γ and η, to be DAL-1/Protein 4.1B-binding proteins. These interactions were verified by using glutathione S-transferase affinity chromatography *in vitro* and co-immunoprecipitation *in vivo*. The interaction of 14-3-3 with DAL-1/Protein 4.1B was specific, as 14-3-3 did not bind to the related Protein 4.1 family members merlin, ezrin or radixin. The DAL-1/Protein 4.1B domain that mediates 14-3-3 binding was mapped to residues Pro^{244} and Leu^{280} within the 4.1/ezrin/radixin/moesin domain. The identification of this novel DAL-1/Protein 4.1B-interacting protein represents the first step towards elucidating its potentially unique mechanism of action.

Key words: membrane, neurofibromatosis 2.

INTRODUCTION

Differentially expressed in adenocarcinoma of the lung (DAL-1)/Protein 4.1B is a tumour suppressor gene belonging to the Protein 4.1 superfamily of proteins, originally isolated using differential display PCR on primary lung tumours [1]. DAL-1/Protein 4.1B represents the second member of the Protein 4.1 family that functions as a tumour suppressor. Merlin or schwannomin, the first identified Protein 4.1 tumour suppressor, is a product of the neurofibromatosis 2 gene [2,3]. Members of the Protein 4.1 family contain several regions of sequence similarity including a 4.1/ezrin/radixin/moesin (FERM) domain consisting of approx. 300 amino acids, and spectrin/actin binding sequences [4]. The FERM domain of the founding family member Protein 4.1R has been found to associate with several membrane proteins, including erythrocyte band 3, calmodulin, glycophorin C, p55 and the chloride-ion channel pICln [5,6]. Similarly, merlin associates with several transmembrane proteins via residues in the N-terminal FERM domain [7,8]. The interaction of merlin with one of these membrane proteins, CD44, has been shown to be critical for merlin growth suppression [7,9].

Similar to merlin, DAL-1/Protein 4.1B localizes to the cell membrane [1] and can function as a negative growth regulator. When DAL-1/Protein 4.1B is reintroduced into DAL-1-deficient lung cancer [1], breast cancer [10] and meningioma [11] cell lines, cell growth is significantly attenuated. Loss of heterozygosity at chromosome 18p11.3, the chromosomal location of DAL-1/Protein 4.1B, is frequent in lung, breast and brain tumours [12]. Detailed analysis of breast cancer tissue samples representing multiple disease stages revealed that 18p11.3 allelic deletions occur in at least 55% of ductal carcinoma *in situ* tumours, suggesting that loss of DAL-1/Protein 4.1B expression occurs early in the progression of this disease [13]. Similarly, the loss of DAL-1/Protein 4.1B expression is detected early in meningioma pathogenesis [14,15].

Although merlin and DAL-1/Protein 4.1B share structural similarities, there are a number of observations that suggest that these two Protein 4.1 tumour suppressors have unique mechanisms of action. First, in cell types where both DAL-1 and merlin are expressed, some tumours exhibit only NF2 loss, whereas others exhibit only DAL-1/Protein 4.1B inactivation [14]. Secondly, overexpression of merlin and DAL-1/Protein 4.1B results in the suppression of meningioma cell line growth, but only merlin overexpression can suppress schwannoma cell growth *in vitro* [11]. Thirdly, DAL-1/Protein 4.1B interacts only with a subset of merlin-binding proteins [11]. And finally, the combined loss of both DAL-1/Protein 4.1B and merlin is more frequently associated with high-grade meningiomas [15].

In an effort to identify unique binding partners for DAL-1/Protein 4.1B that may underlie its mechanism of action as a negative growth regulator, we performed yeast two-hybrid analysis using the 336 residue DAL-1/Protein 4.1B FERM domain. Over 1.5 million co-transfectants from a foetal brain cDNA library were screened to identify several strongly associating proteins, three of which represented the 14-3-3 protein isoforms β, γ and η. These highly conserved and ubiquitously expressed proteins are involved in the regulation of enzyme activity, act as localization anchors for other proteins, and function as adaptor molecules to stimulate protein–protein interactions [16]. The binding of a membrane-associated protein such as DAL-1/Protein 4.1B represents a novel partner for 14-3-3.

Abbreviations used: DAL-1, differentially expressed in adenocarcinoma of the lung; FERM, 4.1/ezrin/radixin/moesin; GST, glutathione S-transferase; NP40, Nonidet P40; TnT, transcription and translation.

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In the present study, we demonstrate the direct association of 14-3-3 with the tumour suppressor protein DAL-1/Protein 4.1B by co-immunoprecipitation *in vivo* and affinity chromatography assays *in vitro*. We further show that the 14-3-3 interaction was specific to DAL-1/Protein 4.1B and not other Protein 4.1 family members. Lastly, we localized the DAL-1/Protein 4.1B–14-3-3 interaction domain to the amino acid residues Pro^{244} and Leu^{280} within the FERM domain.

MATERIALS AND METHODS

Yeast two-hybrid analysis to identify proteins that interact with the FERM domain of DAL-1/Protein 4.1B

Yeast two-hybrid analysis, utilized to identify the proteins interacting with the FERM domain of DAL-1/Protein 4.1B, was performed using the ProQuest™ Two-Hybrid system (Invitrogen, Carlsbad, CA, U.S.A.). The FERM domain of DAL-1/Protein 4.1B, representing the first 336 amino acids of the DAL-1/Protein 4.1B cDNA clone 2A3 (1), was amplified using primers 5′-ACGTGTCGACCATGCAGTGCAAAGTG-ATAC-3′ and 5′-ACGTGCGGCCGCCTCTCCATCCAAGC-TGCG-3′ containing *Sal*I and *Not*I restriction enzyme site linkers respectively. The resulting 1015 bp fragment was cloned into the pDBleu 'bait' vector, creating a fusion protein with a GAL-4 transcription factor DNA-binding domain. A foetal brain cDNA library (Invitrogen) encoding expressed cDNAs fused with the GAL-4 transcription factor activation domain was used for screening. Initially, the library was expanded in Terrific Broth medium (1 litre; 12 g of bacto-tryptone, 24 g of bacto-yeast extract, 4 ml of glycerol) for 16 h at 30 °C, and plasmid DNA was isolated using a Bio-Rad midiprep kit. MaV200 competent cells (Invitrogen) were co-transformed with 10 μg of bait plasmid (pDBLeu-FERM) and 10 μg of library plasmid DNA (pPC86-cDNA). More than 1.5 million cDNA clones were screened, and 14 positively interacting colonies were identified on the basis of growth on leucine-, tryptophan-, uracil- and histidine-lacking plates as well as the ability to process the *lac*Z substrate. To confirm the primary yeast two-hybrid interaction, pPC86-cDNA constructs were isolated from the positive yeast colonies using the Yeast DNA Isolation System (Stratagene, La Jolla, CA, U.S.A.), and inserts were sequenced and analysed with either Thermosequenase Cy5 or Cy5.5 terminator sequencing kits (AP Biotech) on the Long-Read™ System sequencer (Visible Genetics). Homology searches were performed using the BLAST algorithm from the National Center for Biotechnology Information web site (www.nbti.nlm.nih.gov) [17].

Cell culture and Western-blot analysis

The lung tumour cell line NCI-H460 and breast carcinoma cell line T47D were obtained from A.T.C.C. (Manassas, VA, U.S.A.) and maintained in RPMI 1640/10 % foetal calf serum either with insulin (T47D) or without insulin (NCI-H460). The MCF-7 Clone 27 cell line is a DAL-1/Protein 4.1B-inducible cell line generated from the parental MCF-7 cell line using the Ecdysone muristerone-inducible expression kit (Invitrogen) [10]. This cell line as well as parental MCF-7 were grown in MEM with 10 % foetal calf serum, sodium pyruvate, non-essential amino acids and insulin. DAL-1/Protein 4.1B expression is induced by the addition of 1 μM of muristerone to the culture medium for 48 h.

For Western-blot analysis on total cellular protein, lysates were prepared in RIPA buffer [50 mM Tris/150 mM NaCl/0.1 % SDS/0.5 % sodium deoxycholate/1 % Nonidet P40 (NP40)] with protease inhibitors (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Electrophoresis was performed on SDS/PAGE (10 % or 12 % gel) Ready Gels (Bio-Rad) and transferred on to PVDF Plus membrane (MSI Inc.) using the Bio-Rad Mini Protean II transfer system as described previously [1]. For specific extraction of cytoplasmic and membrane protein fractions, cells were resuspended in 20 mM Hepes, 40 mM β-glycerophosphate, 2 mM $MgCl_2$, 1 mM orthovanadate and protease inhibitors (Boehringer Mannheim), and lysed for 10 min at 4 °C. Samples were centrifuged for 30 min at 17000 rev./min. The supernatant, representing the cytosolic fraction, was separated from the pellet, and 2 % NP40 was added. Fresh lysis buffer including 2 % NP40 was added to the pellets representing the cellular membrane fraction. Following incubation for 10 min at 4 °C, samples were centrifuged and the membrane fraction supernatant collected.

In vitro binding assays

pcDNA4 14-3-3β, 14-3-3γ and 14-3-3η cDNA expression constructs were generated by PCR amplification of the coding regions of the 14-3-3 proteins present in the yeast two-hybrid pPC86-cDNA constructs. Amplification primers, containing restriction enzyme linker sequences for *Eco*RI and *Xba*I (underlined), were the following. 14-3-3β forward primer 5′-GGAATTCCCCACGGGAATGACAATGGAT-3′ and 14-3-3β reverse primer 5′-GCTCTAGAGCATTAGTTCTCTCC-CTCCCCA-3′; 14-3-3γ forward primer 5′-GGAATTCCGAG-CCGCGAGCGACATG-3′ and 14-3-3γ reverse primer 5′-GCTCTAGAGCGGCTAATTCTTTCTAATC-3′; and 14-3-3η forward primer 5′-GGAATTCCCGAAGATGGTGGACCG-CGAGC-3′ and 14-3-3η reverse primer 5′-GCTCTAGAGCC-TGGGGCCTTAATTGTTGC-3′. Generated pcDNA4-14-3-3 constructs were verified by sequencing.

Glutathione S-transferase (GST)–DAL-1/Protein 4.1B fusion protein was expressed in BL-21 cells (Novagen) following induction with 0.4 mM isopropyl β-D-thiogalactoside and isolated using the BugBuster™ GST-Bind™ purification kit (Novagen). [^{3}H]Leucine-labelled 14-3-3β, 14-3-3γ and 14-3-3η proteins were generated by a coupled *in vitro* transcription and translation (TnT) kit (Promega, Madison, WI, U.S.A.). For *in vitro* binding assays, individually labelled proteins were confronted with 2 μg of GST–DAL-1 fusion protein using BugBuster binding/wash buffer (4.3 mM Na_2HPO_4/1.47 mM KH_2PO_4/137 mM NaCl/2.7 mM KCl, pH 7.3). Complexes were collected on GST-Bind resin (Novagen), washed three times with Tris-buffered saline/0.3 % (v/v) Tween 20, and separated on SDS/PAGE (10 % gel) Ready Gels (Bio-Rad). Gels were fixed in a propan-2-ol/water/acetic acid (25:65:10) solution, and fluorographic signals were enhanced using Amplify™ (Amersham). Vacuum-dried gels were exposed to autoradiographic film for 1–2 days.

Co-immunoprecipitation

Protein lysates were prepared as described above from the cell lines NCI-H460, T47D and MCF-7 and from MCF-7 Clone 27 before and after a 48 h induction of DAL-1 (residues 1–503) expression with 1 μM of muristerone. Immunoprecipitates were generated from 1–2 mg of lysate using either polyclonal pan anti-14-3-3 (H8; Santa Cruz Biotechnology, New York, NY, U.S.A.), anti-14-3-3β (C-20; Santa Cruz Biotechnology), anti-14-3-3γ (C-16; Santa Cruz Biotechnology) or the polyclonal anti-DAL-1/Protein 4.1B antibody 3A-1. Antibodies were mixed with cell lysates and incubated at 4 °C overnight with rotation. Immunoprecipitates were collected on Protein A–agarose beads (Roche Molecular Biochemicals, Laval, Quebec, Canada), eluted in 2 ×

Laemmli sample buffer, and electrophoresed on SDS/PAGE (10% or 12% gel) Ready Gels (Bio-Rad). Bound proteins were then identified by Western-blot analysis using either anti-14-3-3 antibodies (1:1000) or the anti-DAL-1/Protein 4.1B antibody (1:2000) detected with the mouse IgG or rabbit IgG ImmunStar Chemiluminescence kits (Bio-Rad).

GST affinity chromatography

GST fusion proteins were generated as described previously [11]. Briefly, GST–14-3-3η from the original pPC86 yeast two-hybrid vector (Invitrogen) was cloned into the pGEX.4T-2 vector (Pharmacia), verified by sequencing and transformed into DE3 (BL-21)-competent cells for fusion protein production. Bacterial cultures were induced overnight with 0.5 mM isopropyl β-D-thiogalactoside at room temperature, lysed in NP40 lysis buffer and the GST fusion proteins isolated on glutathione–agarose beads (Sigma) for the interaction experiments. Each fusion protein was newly isolated for each experiment and verified by SDS/PAGE with Coomassie Blue visualization.

DAL-1/Protein 4.1B, merlin, ezrin (N-ezrin, C-ezrin) and radixin (mer-rad and rad-mer) were generated by reverse transcriptase PCR as described previously [18]. Rad-mer is a hybrid protein comprised of the N-terminal radixin sequences (1–323) joined in frame with C-terminal merlin sequences (341–583), whereas mer-rad is composed of N-terminal merlin (1–341) and C-terminal radixin sequences (324–583). DAL-1/Protein 4.1B constructs, schematically shown in Figure 5(A), were created using full-length DAL-1/Protein 4.1B as a template and the TA cloning kit (Invitrogen) according to the manufacturer's instructions. Construct Met^{1} was generated by PCR using primers that amplify the FERM domain alone (FermF: 5′-GCA-CTA-GTC-AAG-GAC-TCA-GAA-GGG-GTA-G-3′ and FermR: 5′-TCA-TGC-AGC-AGA-CAT-AGA-ATC-CTT-C-3′). Constructs Lys^{195}, Pro^{244} and Leu^{280} were generated by PCR using the reverse primer U2R (5′-TCA-CTC-AGT-GGC-GGT-GGT-CTC-3′) and unique forward primers (K195F: 5′-CAC-TAG-TCC-CGG-GAG-AGT-TTG-AAC-AAT-TTG-3′, P244F: 5′-GCA-CTA-GTC-CTG-TTA-CCA-GAA-GCA-CCT-CCC-3′ and L280F: 5′-TCA-CTT-GGG-AGG-TGC-TTC-TGG-TAA-CAG-3′). Constructs $Met^{1–244}$ and $Met^{1–280}$, encompassing the first 244 and 280 amino acids respectively of the FERM domain of DAL-1, were generated by PCR using the forward primer FermF and unique reverse primers (P244R: 5′-TCA-CGG-CCG-GAT-CTT-AAT-GTA-AAA-GTT-3′ and L280R: 5′-TCA-CAG-TAG-TCT-GAA-AAA-TGT-ATG-ATG-3′). The constructs were verified by sequencing, and then subcloned into the pcDNA3 vector (Invitrogen). Proteins were synthesized by the *in vitro* coupled TnT method (Promega; catalog no. L4610) in the presence of [^{35}S]methionine according to the manufacturer's protocol and confirmed by SDS/PAGE and autoradiography. Interaction experiments were performed by incubating 5 μl of the TnT reaction containing the radiolabelled proteins with equimolar amounts of GST fusion proteins immobilized on glutathione beads in a total volume of 1 ml of TEN buffer [10 mM Tris (pH 7.5)/150 mM NaCl/5 mM EDTA/1% (v/v) Triton X-100] for 4 h at 4 °C. The bound agarose beads were then washed four times in TEN buffer. For assays using protein fragments $Met^{1–244}$ and $Met^{1–280}$, radiolabelled proteins in 5 μl of TnT reaction buffer were incubated with equimolar amounts of GST fusion proteins immobilized on glutathione beads in a total volume of 1 ml of NET buffer [25 mM Tris (pH 7.5)/100 mM NaCl/3 mM EDTA] containing 1% BSA for 4 h at 4 °C. Beads were washed twice with NET buffer plus 0.1% (v/v) Triton X-100 and twice with NET buffer plus 0.05% (v/v) Triton X-100. An aliquot of the unbound fraction was saved for all the assays. The unbound and bound fractions were eluted in Laemmli buffer, boiled for 5 min, separated by SDS/PAGE and analysed by autoradiography. Identical results were obtained with either NET or TEN buffers. In all experiments, significant binding to immobilized GST alone was not observed. Each interaction experiment has been repeated at least three times.

RESULTS

DAL-1/Protein 4.1B interacts with members of the 14-3-3 family

Yeast two-hybrid interaction cloning was performed to isolate potential binding partners of the tumour suppressor protein DAL-1/Protein 4.1B. The FERM domain of DAL-1/Protein 4.1B (Met^{1}-Gly^{336}) was fused in frame with the DNA-binding domain of GAL4 in the pDBleu bait vector and used to screen a human foetal brain cDNA library. The high expression of DAL-1/Protein 4.1B in the brain [1] suggests that physiologically relevant binding partners would be represented in this library. Analysis of more than 1.5 million co-transfectants led to the isolation of a total of 14 positive clones after two rounds of screening. Three of these colonies (#4, #14 and #41 in Figure 1) contained cDNAs encoding the 14-3-3β, 14-3-3γ and 14-3-3η isoforms. The cDNA insert sizes ranged from 1.6 to 4.0 kb and sequence analysis confirmed representation of the entire coding region of these 14-3-3 proteins in the cDNA isolates. To confirm the interaction between DAL-1/Protein 4.1B and 14-3-3 in mammalian cell extracts, co-immunoprecipitation experiments were performed using protein lysates from the endogenously expressing non-small cell lung cancer cell line (NCI-H460), DAL-1/Protein 4.1B-null breast cancer cell line (MCF-7) and

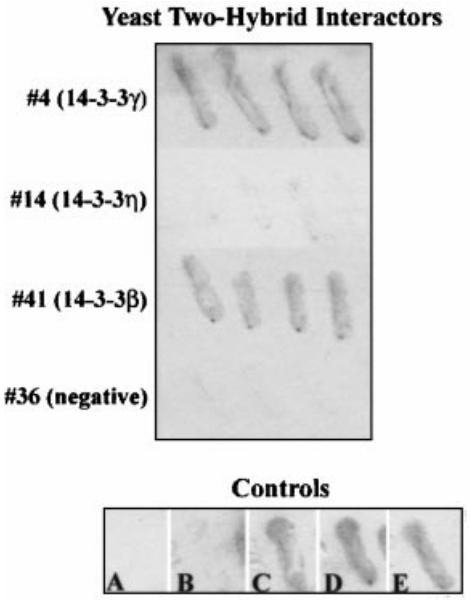

Figure 1 14-3-3 interacts with the FERM domain of DAL-1/Protein 4.1B

A foetal brain cDNA library was screened with the FERM domain of DAL-1/Protein 4.1B as bait. The resulting co-transfectants exhibiting interaction were selected in part for their ability to induce transcription of the *lacZ* gene resulting in a blue colour when assayed with 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside. Co-transfectants #4, #14 and #41 were positive for *lacZ* expression, whereas #36 was negative. Controls representing known protein interactions of varying strength were plated for comparison. (**A**) No cDNA insert control; (**B**) RB/E2F, weak interaction; (**C**) *Drosophila* DP/E2F, moderate interaction; (**D**) Fos/Jun, strong interaction; (**E**) GAL4-binding domain/GAL4-activation domain, very strong interaction.

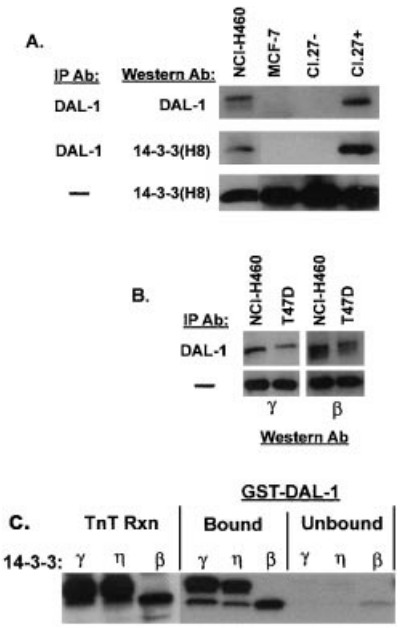

Figure 2 DAL-1/Protein 4.1B interacts directly with 14-3-3β, 14-3-3η and 14-3-3γ in mammalian cell extracts

(**A**) DAL-1/Protein 4.1B and 14-3-3 proteins from NCI-H460, MCF-7 and the DAL-1-inducible cell line Clone 27 were tested for their ability to co-immunoprecipitate. Products immunoprecipitated with the polyclonal anti-DAL-1/Protein 4.1B antibody 3A-1 were analysed by Western-blot analysis with either the DAL-1/Protein 4.1B antibody or the polyclonal H8 anti-14-3-3 antibody. Analysis showed that endogenous DAL-1/Protein 4.1B protein (from NCI-H460) and DAL-1 protein induced after a 48 h exposure to 1 μM of muristerone (Cl.27 +) was able to co-immunoprecipitate with 14-3-3 proteins. Cell lines negative for DAL-1/Protein 4.1B expression (MCF-7 and Cl.27 —) did not show interaction with 14-3-3. The endogenous level of 14-3-3 proteins in these cell lines is shown in the lower panel. (**B**) Endogenously expressed DAL-1/Protein 4.1B was examined for its ability to interact specifically with the endogenous β and γ isoforms of 14-3-3 in NCI-H460 and T47D. Western-blot analysis with 14-3-3β or 14-3-3γ-specific antibodies following immunoprecipitation with DAL-1/Protein 4.1B antibody 3A-1 revealed a strong band for each isoform, indicating that DAL-1/Protein 4.1B and 14-3-3 proteins can interact. (**C**) GST–DAL-1 fusion protein (2 μg) was generated and interacted *in vitro* with radiolabelled 14-3-3β, 14-3-3γ and 14-3-3η. Aliquots of each radiolabelled protein from the *in vitro* TnT reactions were run as a reference (TnT rxn). Equivalent amounts of protein collected on GST beads and remaining in the supernatant were analysed by electrophoresis and autoradiography. All three 14-3-3 isoforms tested directly interacted with the GST–DAL-1 fusion protein. IP, immunoprecipitation; Ab, antibody.

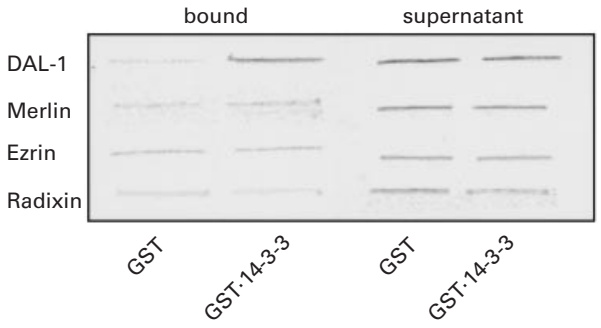

Figure 3 14-3-3 binds specifically to DAL-1/Protein 4.1B

Binding of 14-3-3 to DAL-1/Protein 4.1B, merlin, ezrin and radixin was investigated by GST affinity interactions using GST alone or GST–14-3-3η as described in the Materials and methods section. The bound and supernatant fractions are shown for each representative interaction. DAL-1/Protein 4.1B exhibited significant binding to 14-3-3. Binding to merlin, ezrin or radixin (rad-mer, results shown; mer-rad, results not shown) was not observed.

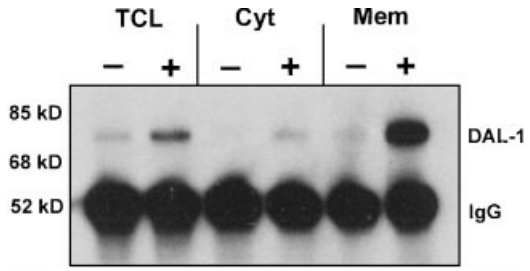

Figure 4 14-3-3 complexes with DAL-1/Protein 4.1B at the plasma membrane

Protein lysates were isolated from the DAL-1/Protein 4.1B-inducible cell line Clone 27, before and after 48 h exposure to the expression-inducing agent muristerone. Total cell lysate (TCL) or protein isolated from fractions representing plasma membrane (Mem) or cytoplasmic (Cyt) components were immunoprecipitated using the 14-3-3γ antibody, followed by Western-blot analysis with the polyclonal DAL-1/Protein 4.1B antibody 3A-1. Results show that DAL-1 protein expression is significantly induced upon exposure to muristerone. The majority of DAL-1 protein produced can be immunoprecipitated from the membrane component by the 14-3-3 antibody, indicating that 14-3-3 proteins are found complexed with DAL-1 proteins at the plasma membrane. IgG, immunoglobulin heavy chain; kD, kilodalton; —, without induction of DAL-1 protein expression; +, with induction of DAL-1 protein expression.

a DAL-1 inducible MCF-7-derived cell line (Clone 27), in which expression of DAL-1 (residues 1–503) is induced after treatment with 1 μM muristerone in culture [10]. As shown in Figure 2(A), all cell lines expressed abundant levels of 14-3-3 proteins, whereas only NCI-H460 and the DAL-1-inducible MCF-7 Clone 27 (Cl.27+) showed a detectable DAL-1/Protein 4.1B protein. Immunoprecipitation using the polyclonal anti-DAL-1/Protein 4.1B antibody 3A-1 followed by Western-blot analysis of 14-3-3 proteins using the monoclonal pan-14-3-3 (H8) antibody demonstrated an interaction in mammalian cell extracts only when the Protein 4.1B/DAL-1 protein was concurrently expressed (Figure 2A). Specific isoform binding was examined by using available monoclonal antibodies against 14-3-3γ (C-16) and 14-3-3β (C-20). As shown in Figure 2(B), 14-3-3β and 14-3-3γ were equally expressed in NCI-H460 and the endogenous DAL-1/Protein 4.1B-expressing breast cancer cell line T47D. In both cases, 14-3-3γ and 14-3-3β co-immunoprecipitated with DAL-1/Protein 4.1B, indicating this tumour suppressor protein has a generalized capability to interact with various 14-3-3 isoforms.

In vitro binding assays were also performed to reveal whether these proteins interacted in a direct manner or required the presence of other cellular proteins. Bacterially generated GST–DAL-1 fusion protein (residues 1–503) was incubated with either radiolabelled 14-3-3β, 14-3-3γ or 14-3-3η produced by *in vitro* coupled TnT (Figure 2C). Complexed proteins were retrieved on GST affinity beads, after which the presence of radiolabelled 14-3-3 protein was analysed by SDS/PAGE separation and autoradiography. As shown in Figure 2(C), significant binding of [^{3}H]-14-3-3β, [^{3}H]-14-3-3γ and [^{3}H]-14-3-3η to the GST–DAL-1 fusion protein was observed, suggesting that DAL-1/Protein 4.1B directly interacts with 14-3-3 proteins. No significant binding to GST protein alone was found (results not shown).

14-3-3 binding is specific to DAL-1/Protein 4.1B

To determine whether the 14-3-3 interaction was unique to DAL-1/Protein 4.1B or was observed with other members of the

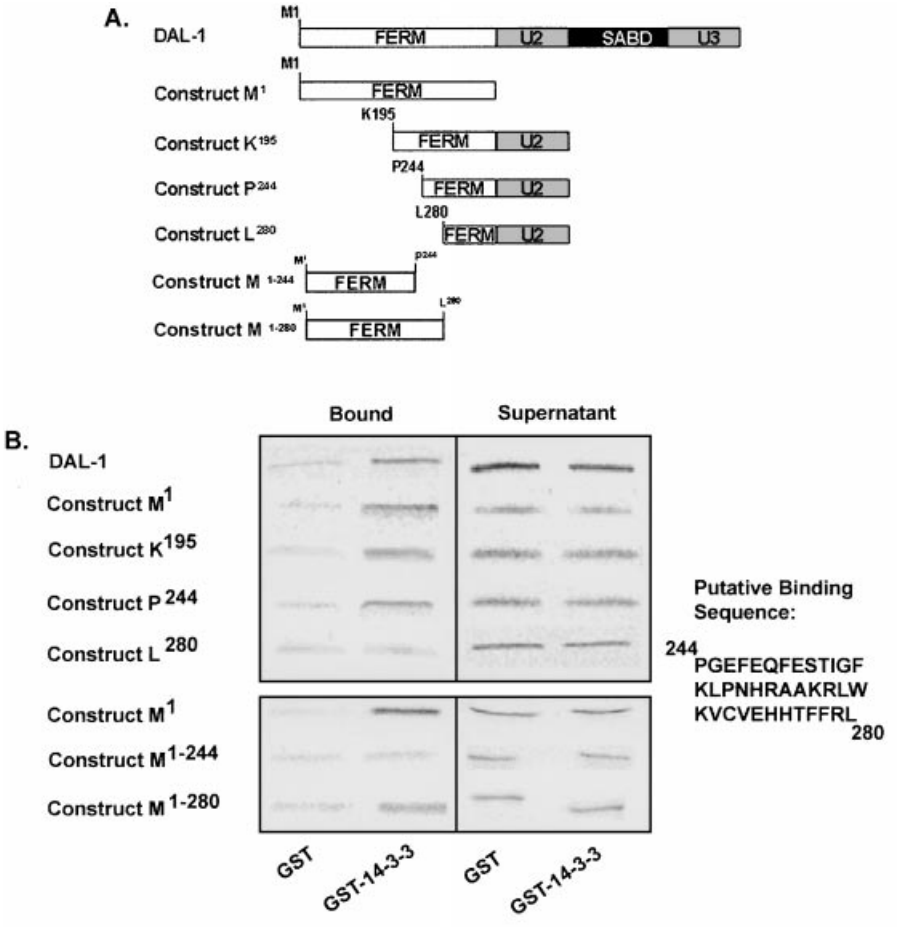

Figure 5 Definition of the 14-3-3-binding domain

(**A**) Schematic of the domains present in full-length DAL-1/Protein 4.1B and constructs designed to determine the minimum 14-3-3-binding domain. Letters and numbers correspond to the first amino acid residue of DAL-1/Protein 4.1B and the constructs (e.g. Met^1 corresponds to the initiating methionine of DAL-1/Protein 4.1B). $Met^{1–244}$ and $Met^{1–280}$ represent N-terminal protein fragments of the FERM domain alone. (**B**) 14-3-3 binding to DAL-1, Met^1, Lys^{195}, Pro^{244}, Leu^{80}, $Met^{1–244}$ and $Met^{1–280}$. GST affinity interactions were performed using GST alone or GST–14-3-3η. The bound and supernatant fractions are shown for each representative interaction. DAL-1/Protein 4.1B, Met^1, Lys^{195}, Pro^4 and $Met^{1–280}$ exhibited considerable binding to 14-3-3. Significant binding to GST alone, Leu^{280} or $Met^{1–244}$ was not observed. The 37-amino-acid sequence of DAL-1/Protein 4.1B, hypothesized to comprise the putative 14-3-3-binding sequence, is shown at the bottom right.

Protein 4.1 family, *in vitro* interaction experiments were next performed using bacterial GST–14-3-3η and radiolabelled Protein 4.1 family members. Consistent with the results obtained from the yeast two-hybrid and co-immunoprecipitation assays, 14-3-3 interacts specifically with DAL-1/Protein 4.1B (Figure 3), but not to the Protein 4.1 family members merlin, ezrin or radixin. The selectivity of the 14-3-3 interaction suggests that such specific partnerships may be responsible for defining potential unique growth-regulatory functions for DAL-1/Protein 4.1B.

14-3-3 is complexed with DAL-1/Protein 4.1B at the plasma membrane

As 14-3-3 proteins are abundantly expressed cytoplasmic proteins with an affinity for a variety of cellular proteins, we asked whether 14-3-3 was bound to DAL-1/Protein 4.1B at the plasma membrane. To address this question, cell lysates were generated from the MCF-7 Clone 27 inducible cell line before and after induction of DAL-1 protein expression. Lysates were immunoprecipitated with the 14-3-3γ antibody and analysed by Western blotting using the DAL-1/Protein 4.1B antibody. As shown in Figure 4, DAL-1/Protein 4.1B protein expression was induced in the MCF-7 Clone 27 cell line after incubation with 1 μM of muristerone. Isolation of plasma membrane proteins followed by immunoprecipitation revealed that 14-3-3γ was complexed with the DAL-1 protein at the plasma membrane. This co-localization suggests that a significant proportion of 14-3-3 protein is bound to DAL-1 at the plasma membrane. Reciprocal immunoprecipitation experiments with the DAL-1/Protein 4.1B antibody followed by Western-blot analysis with 14-3-3γ or 14-3-3β were also attempted; however, buffer conditions used for cellular fractionation precluded the ability of the DAL-1/Protein 4.1B antibody to function effectively in the immunoprecipitation reactions.

14-3-3 interaction requires specific FERM domain sequences

The use of the isolated FERM domain (Met^1-Gly^{336}) of DAL-1/Protein 4.1B in the initial yeast two-hybrid screen suggested that this region contains a 14-3-3-binding sequence. To define the DAL-1/Protein 4.1B sequence that mediates the association with 14-3-3, we generated a series of deletion constructs that remove specific regions of the FERM domain of DAL-1/Protein 4.1B.

These constructs, cloned into pcDNA3 and used to generate molecules for *in vitro* TnT, include the entire FERM domain (Met^{1}) as well as sequential N-terminal FERM domain deletions, designated Lys^{195}, Pro^{244} and Leu^{280} (Figure 5A). Radiolabelled DAL-1/Protein 4.1B protein fragments and the 503 residue DAL-1 molecule were incubated with GST or GST–14-3-3η and assayed for their ability to interact *in vitro* (Figure 5B). In these experiments, DAL-1 and constructs with a deletion of the first 195 or 244 amino acids maintained the ability to complex with GST–14-3-3η. However, deletion of the amino acids between Pro^{244} and Leu^{280} abolished 14-3-3η binding, suggesting that this 37-amino-acid region (Pro^{244}-Leu^{280}) contains residues critical for 14-3-3 binding. To determine this possibility and to exclude the possibility that carboxyl-terminal sequences in the U2 region also participate in 14-3-3 binding, we generated two additional constructs that include only FERM domain sequences ($Met^{1–244}$ and $Met^{1–280}$; Figure 5A) and analysed their ability to interact with 14-3-3η *in vitro*. Only the $Met^{1–280}$ FERM fragment retained the ability to complex with 14-3-3 (Figure 5B). Collectively, these results suggest that the FERM domain residues Pro^{244}-Leu^{280} are sufficient to mediate the binding of DAL-1/Protein 4.1B to 14-3-3. The sequence of this putative binding domain is shown in Figure 5(B). A conventional 14-3-3-binding site, represented by consensus sequence of RSXpSXP [19], is not found within this 37-amino-acid stretch. Amino acid analysis also failed to identify homology to any of the other previously reported 14-3-3-binding motifs [20], suggesting that this interaction involves a novel interaction sequence.

DISCUSSION

Previous studies have demonstrated the tumour suppressor properties of the DAL-1/Protein 4.1B molecule [1,10,11]. In an effort to identify unique binding partners for DAL-1/Protein 4.1B that may transduce its negative cell-growth-regulatory signal, we utilized a yeast two-hybrid interaction assay with the DAL-1/Protein 4.1B FERM domain as bait. This screen identified the 14-3-3 adaptor protein as a DAL-1/Protein 4.1B-interacting molecule. We demonstrated that 14-3-3 does not associate with other Protein 4.1 family members including merlin, ezrin and radixin. The DAL-1/Protein 4.1B–14-3-3 interaction was confirmed both *in vitro* and *in vivo*, and appeared to be a direct association that does not require additional protein partners. The region of 14-3-3 binding within the DAL-1/Protein 4.1B FERM domain localized to a specific 37-amino-acid sequence that does not contain traditional 14-3-3-binding motifs.

14-3-3 proteins consist of seven primary isoforms (β, γ, ϵ, ζ, η, τ and θ) with additional isoforms α and δ representing phosphorylated forms of β and ζ respectively [21]. These proteins exist as dimers, forming two internal pockets with which other proteins can interact [22]. In our studies, we found that all 14-3-3 molecules examined were able to bind to DAL-1/Protein 4.1B. The association between DAL-1/Protein 4.1B and 14-3-3 appears to be direct, based on *in vitro* affinity chromatography binding experiments, although other proteins may subtly facilitate or regulate this interaction *in vivo*.

14-3-3 molecules typically bind to proteins that contain traditional consensus binding motifs, such as RSXpSXP, although other binding motifs have been reported. For example, protein phosphatase PTPH1, a related Protein 4.1 family member, has been shown to bind 14-3-3β via two C-terminal motifs, $RSLS^{359}VE$ and $RVDS^{853}EP$ [23]. Using a series of FERM deletion constructs, we narrowed the DAL-1/Protein 4.1B–14-3-3-binding site to a 37-amino-acid domain that lacks previously reported 14-3-3-binding motifs. BLAST searches failed to identify other proteins with this 37 amino acid domain, aside from other known FERM-containing molecules. In this regard, the DAL-1/Protein 4.1B FERM interaction sequence is unique [16,19,20].

Phosphorylation has been demonstrated to be an important modification for proteins interacting with 14-3-3 isoforms. For example, the association between 14-3-3 and PTPH1 is dependent on serine phosphorylation [23]. Likewise, the activation of the Na^{+}/H^{+} exchanger-1 protein, which interacts with 14-3-3 using the motif RIGSDP, is also dependent on the phosphorylation of the serine within this motif [24]. However, phosphorylation-independent peptide binding of 14-3-3 also occurs. 14-3-3ϵ binds to calmodulin, a known interactor of several DAL-1/Protein 4.1B-related Protein 4.1 family molecules, in a non-phosphorylation-dependent manner [25]. Therefore molecules like DAL-1/Protein 4.1B might use non-traditional and phosphorylation-independent interaction mechanisms to interact with 14-3-3. Studies are underway to determine whether 14-3-3 binding is influenced by DAL-1/Protein 4.1B phosphorylation *in vivo*.

The interaction of DAL-1/Protein 4.1B with 14-3-3 is not a ubiquitous characteristic of all members of the Protein 4.1 family. Our studies demonstrated that the FERM domains of merlin, ezrin and radixin were not capable of associating with 14-3-3. These results strongly suggest that 14-3-3 may represent a unique interactor specific to the DAL-1/Protein 4.1B tumour suppressor. Previous studies from our laboratories have demonstrated that although merlin and DAL-1/Protein 4.1B are structurally similar molecules, they differ in their ability to interact with specific proteins [11]. Merlin has been shown to bind to several proteins that reside at the cell membrane, including CD44 and β1-integrin [7,8]. The association of merlin with CD44 is required for merlin growth suppression [7,9]. In addition, merlin associates with the hepatocyte growth-factor-regulated tyrosine kinase substrate [26], Na^{+}/H^{+} exchanger regulatory factor [27], syntenin [28] and schwannomin-interacting protein 1 [29]. To date, we have shown that DAL-1/Protein 4.1B does not interact *in vitro* with either the hepatocyte growth-factor-regulated tyrosine kinase substrate or schwannomin-interacting protein 1 and lacks the binding domain required for syntenin binding ([11]; V. A. Robb and D. H. Gutmann, unpublished work). Collectively, these results suggest that merlin and DAL-1/Protein 4.1B are unique molecules important for growth regulation.

Although 14-3-3 is described as a cytoplasmic protein, we have shown here that 14-3-3 isoforms associate with DAL-1/Protein 4.1B at the cell membrane. It is possible that DAL-1/Protein 4.1B recruits 14-3-3 to the cell membrane, allowing it to interact with specific effector proteins to transduce a growth suppressor signal. A similar mechanism has been proposed for 14-3-3 binding to RAF [30–32]. Alternatively, 14-3-3 proteins may be guiding DAL-1/Protein 4.1B to the plasma membrane, as has been suggested for other 14-3-3-interacting proteins [16,19]. In either case, understanding the relationship between DAL-1/Protein 4.1B and 14-3-3 is likely to yield important insights into the mechanisms that regulate DAL-1/Protein 4.1B function.

Several other functions have been attributed to 14-3-3 molecules, including regulation of signal transduction through c-*Cbl* [33] and phosphoinositide 3-kinase [34]. One intriguing 14-3-3-binding partner is the pro-apoptotic protein, Bcl-2/Bcl-X_L-antagonist, causing cell death (BAD) [35]. 14-3-3 binds to phosphorylated BAD and prevents it from translocating to the mitochondria to initiate programmed cell death or apoptosis [36,37]. Recent work [10] from our laboratories has shown that DAL-1/Protein 4.1B overexpression results in apoptosis in breast cancer cell lines. At present, the relationship between DAL-1/Protein 4.1B, programmed cell death and 14-3-3 binding is

actively being investigated. These analyses will require a better understanding of the specific residues in DAL-1/Protein 4.1B that are needed for mediating 14-3-3 binding. Experiments in our laboratory demonstrate that the 37-amino-acid region necessary for 14-3-3 binding overlaps with regions that not only mediate other critical DAL-1/Protein 4.1B associations but also specify the correct subcellular localization of DAL-1/Protein 4.1B to the plasma membrane (D. H. Gutmann and I. F. Newsham, unpublished work). Experiments are underway to identify specific residues within the FERM domain whose alteration selectively impairs DAL-/Protein 4.1B binding to 14-3-3 without affecting other binding proteins. These mutants will be critical to deciphering the functional significance of the 14-3-3 association to DAL-1/Protein 4.1B regulation of cell growth.

We thank Dr Oliver Bögler for help with the yeast two-hybrid analysis and W. Li and A. C. Hirbe for expert technical assistance. This work was supported by grants NS/CA41520 and NCI/CA77730 to D.H.G. and I.F.N. respectively and by the generosity of the Hermelin Brain Tumor Center donors.

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Received 9 January 2002/15 April 2002; accepted 25 April 2002
Published as BJ Immediate Publication 25 April 2002, DOI 10.1042/BJ20020060