Protein 4.1B/Differentially Expressed in Adenocarcinoma of the Lung-1 Functions as a Growth Suppressor in Meningioma Cells by Activating Rac1-Dependent c-Jun-NH_2-kinase Signaling

Mark A. Gerber, Scott M. Bahr, and David H. Gutmann

Department of Neurology, Washington University School of Medicine, St. Louis, Missouri

Abstract

Meningiomas are the second most common brain tumor in adults, yet comparatively little is presently known about the dysregulated growth control pathways involved in their formation and progression. One of the most frequently observed genetic changes in benign meningioma involves loss of protein 4.1B expression. Previous studies from our laboratory have shown that protein 4.1B growth suppression in meningioma is associated with the activation of the c-Jun-NH_2-kinase (JNK) pathway and requires localization of a small unique region (U2 domain) of protein 4.1B to the plasma membrane. To define the relationship between protein 4.1B expression and JNK activation, as well as to determine the mechanism of JNK activation by protein 4.1B, we used a combination of genetic and pharmacologic approaches. In this report, we show that protein 4.1B/differentially expressed in adenocarcinoma of the lung-1 (DAL-1) expression in meningioma cells *in vitro* results in JNK activation, which requires the sequential activation of Src, Rac1, and JNK. In addition, inhibition of Rac1 or JNK activation abrogates protein 4.1B/DAL-1 growth suppression and cyclin A regulation. Last, protein 4.1B/DAL-1 regulation of this critical growth control pathway in meningioma cells requires the presence of the U2 domain. Collectively, these observations provide the first mechanistic insights into the function of protein 4.1B as a growth regulator in meningioma cells. (Cancer Res 2006; 66(10): 5295-303)

Introduction

Although meningiomas are among the most common nervous system tumors, the specific genetic changes that are critical for their formation and progression are poorly understood. Of the genetic events known to be associated with meningioma tumorigenesis, chromosome 22q loss of heterozygosity with *NF2* gene inactivation is the most commonly detected alteration (1–5). Merlin, the protein product of the *NF2* gene, is a member of the protein 4.1 family, based on structural homology (6, 7). Protein 4.1 family members are thought to function at the plasma membrane and are hypothesized to link plasma membrane and cytoskeleton processes (8). Roles for this class of proteins in cell morphogenesis, membrane structure, cell adhesion, and the regulation of cell growth have been well documented (9).

Recent studies from our laboratory and others have implicated another member of the protein 4.1 superfamily, protein 4.1B, in the molecular pathogenesis of a wide variety of diverse human cancers (10–13), including meningiomas (10, 14, 15). In these reports, protein 4.1B loss has been observed in human meningiomas at the DNA level by fluorescence *in situ* hybridization and PCR-based loss of heterozygosity (14). Protein 4.1B loss has also been shown at the RNA level by reverse transcription-PCR and at the protein level by Western blot and immunohistochemistry (14, 15). Protein 4.1B loss was found to represent an early event in meningioma formation and was observed in 50% to 60% of WHO grade I benign meningioma (15). Moreover, reexpression of protein 4.1B or a smaller fragment of the entire protein, termed differentially expressed in adenocarcinoma of the lung-1 (DAL-1; ref. 10), resulted in meningioma cell growth suppression (16, 17).

Structural analysis of protein 4.1B reveals that it is composed of a highly conserved NH_2-terminal FERM (4.1, ezrin, radixin, moesin) domain, a spectrin-actin binding domain, a relatively conserved COOH-terminal domain, and three interspersed regions of unique sequence identity (termed U1, U2, and U3; refs. 9, 18). Whereas protein 4.1B contains unique amino acid sequences not found in the DAL-1 molecule, it has been established that DAL-1 contains all the residues necessary for the tumor suppressor properties of protein 4.1B (10, 16). Recently, we reported that protein 4.1B/DAL-1 growth suppression in meningioma cells requires only the U2 domain, when properly localized to the plasma membrane (17). Similarly, deletion of the U2 domain from the DAL-1 protein results in a loss of meningioma cell growth suppression, underscoring the importance of this region in protein 4.1B function.

Although the molecular mechanisms underlying protein 4.1B/DAL-1 regulation of cell growth are not clear, we have previously shown that DAL-1 reexpression in protein 4.1B–deficient IOMM-Lee meningioma cells results in c-Jun-NH_2-kinase (JNK) activation (17). In the present report, we show that DAL-1/protein 4.1B specifically activates the JNK signaling cascade and that this activation relies on the presence of the U2 domain. Further, we show that DAL-1 expression results in reduced cyclin A expression and hyperphosphorylation of the retinoblastoma (Rb) protein, which are hallmarks of reduced cell proliferation, and that these effects require JNK activation. Using a combination of genetic and pharmacologic approaches, we show that protein 4.1B/DAL-1 JNK-mediated growth suppression in meningioma cells requires activation of Src, Rac1, and mixed-lineage kinase 3 (MLK3). Collectively, these data provide the first molecular insights into the mechanism of growth regulation by the protein 4.1B tumor suppressor.

Materials and Methods

Cell lines. Human IOMM-Lee meningioma cells were maintained in complete DMEM plus 10% FCS, as previously described (19). IOMM-Lee meningioma cell lines stably expressing empty pcDNA3, pcDNA3.DAL-1,

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

Requests for reprints: David H. Gutmann, Department of Neurology, Washington University School of Medicine, Box 8111, 660 South Euclid Avenue, St. Louis, MO 63110. Phone: 314-362-7379; Fax: 314-362-2388; E-mail: gutmannd@neuro.wustl.edu.

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and pcDNA3.DAL-1 ΔU2 were maintained in complete DMEM plus 10% FCS and 500 μg/mL G418, as previously described (17). Inducible IOMM-Lee cells were generated in two steps. First, IOMM-Lee cells were cotransfected with pBabe.puro and pUHG17.1 containing the rtTA open-reading frame followed by an IRES-Luciferase cDNA fragment (generously provided by Dr. David Piwnica-Worms, Washington University, St. Louis, MO). Transfected cells were selected in puromycin (0.5 μg/mL) for 3 weeks, and clones were assayed for luciferase activity to select the best expressers. Clonal cells from these selections were then transfected with a pTRE2hyg-Myc.protein 4.1B-IRES-enhanced green fluorescent protein (EGFP) construct (pTRE2hyg-Myc was obtained from BD Biosciences, Palo Alto, CA), and were selected in hygromycin (200 μg/mL). Surviving clones were then treated with 2 μg/mL doxycycline and screened for EGFP expression. Western blot analysis with anti-DAL-1 or 9E10 myc monoclonal antibodies was used to confirm 4.1B expression. Resulting cell lines were maintained in tetracycline-free complete DMEM, with 10% FCS, puromycin (0.5 μg/mL), and hygromycin (200 μg/mL).

Inhibitors. Inhibitors for JNK (JNK inhibitor I, 2 μmol/L), MLK3 (K252a, 200 nmol/L), Src (genistein, 5 μmol/L; PP2, 10 nmol/L), and the negative control for PP2 (PP3, 10 nmol/L) were purchased from Calbiochem (San Diego, CA). All inhibitor treatments were done for 48 hours before cell harvest and lysis.

Western blots. Cells were lysed in NP40 lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.5% NP40, 1 mmol/L DTT] plus protease inhibitors (leupeptin, benzamidine, phenylmethylsulfonyl fluoride, and aprotinin) and phosphatase inhibitor (1 mmol/L orthovanadate) on ice. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL), and samples were boiled in Laemmli buffer. Thirty micrograms protein from each sample were used for Western blot analysis using the following antibodies: anti-c-myc, 9E10 clone (Sigma, St. Louis, MO, 1:1,000 dilution); anti-α-tubulin (Sigma, 1:20,000 dilution); anti-14-3-3 (Santa Cruz Biotechnologies, Santa Cruz, CA, 1:10,000 dilution); anti-DAL-1 polyclonal (1:2,000 dilution; ref. 10); anti-phospho (p)-Rb (sc-50, Santa Cruz Biotechnologies, 1:500 dilution); anti-Rac1, clone 23A8 (Upstate Biotechnologies, Lake Placid, NY); and anti–cyclin A (Santa Cruz Biotechnologies, 1:1,000 dilution). The antibodies used for the signaling studies were all purchased from Cell Signaling Technology (Beverly, MA): anti-Akt and anti-p-Akt (Thr^{308}; 1:1,000 dilution); anti-S6 and anti-p-S6 ($Ser^{240/244}$; 1:1,000 dilution), anti-S6 kinase and anti-p-S6 kinase (Thr^{389}; 1:1,000 dilution), anti-p42/p44 mitogen-activated protein kinase (MAPK) and p-MAPK (Thr^{202}/Tyr^{204}; 1:1,000 dilution); anti-Src and anti-p-Src (Tyr^{416}; 1:1,000 dilution); anti-MLK3 and anti-p-MLK3 (Thr^{277}/Ser^{281}; 1:1,000 dilution); anti-JNK and anti-p-JNK (Thr^{183}/Tyr^{185}; 1:1,000 dilution); anti-p38 and anti-p-p38 (Thr^{180}/Tyr^{182}; 1:1,000 dilution); and anti–apoptosis signal-regulating kinase 1 (ASK-1) and anti-p-ASK1 (Thr^{845}; 1:1,000 dilution).

Rac1/cdc42 activation assay. Rac1 activation assays were carried out using the Rac Activation Assay kit (Upstate Biotechnologies). Subconfluent cells (~60% confluent), from each stable cell line or transiently transfected, were washed 3× in ice-cold 1× TBS. Cells were collected by scraping in 1× MLB [Mg^{2+} lysis/wash buffer; 25 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% Igepal CA-630, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, and 10% glycerol] with a rubber policeman and lysed by pipetting. After adequate triturition, lysates were incubated on ice for 5 minutes, and then cleared by centrifugation at 5,000 rpm for 5 minutes in a table top microcentrifuge at 4°C. After removal of an aliquot of each lysate to represent the input fraction, the lysates were transferred to new Eppendorf tubes, and 10 μL Pak1 PBD-agarose suspension was added. The samples were then rocked gently for 1 hour at 4°C, and the agarose conjugates were pelleted by a 5-second pulse centrifugation at maximum speed (14,000 rpm) in a microcentrifuge. Conjugates were washed thrice with ice-cold MLB, and were then boiled in 2× Laemmli buffer for 5 minutes. Suspensions and input samples were separated by 15% SDS-PAGE, and Western blot analysis was used to detect Rac1 and DAL-1 in the input fraction and activated Rac1 in the bound fraction. For positive and negative controls, lysates were incubated in 10 mmol/L EDTA and either 0.1 mmol/L GTPγS (positive) or 1 mmol/L GDP (negative) for 15 minutes at 30°C before binding to PBD-agarose conjugates. Binding reactions for the positive and negative controls were stopped by the addition of $MgCl_2$ to 60 mmol/L before binding. For p-Src and p-JNK inhibitor experiments, subconfluent cells were treated with the inhibitors for 48 hours before harvest. Each experiment was done at least thrice with identical results. Activation of cdc42 was measured using the same methods described for Rac1 activation, except that Western blots were probed with an anti-Cdc42 antibody (Cell Signaling Technology, 1:500 dilution).

Rap1 activation assay. Cells were grown to ~60% confluency in 100 mm dishes, and were harvested by scraping in MAPK lysis buffer [20 mmol/L Tris (pH 7.5), 10 mmol/L EGTA, 40 mmol/L β-glycerolphosphate, 1% NP40, 2.5 mmol/L $MgCl_2$, and 20 mmol/L $NaVO_4$] plus protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and benzamidine). After a 10-minute incubation on ice, lysates were cleared by centrifugation at 10,000 × *g* for 10 minutes at 4°C. After removal of an aliquot to represent the input fraction, lysates were incubated with 20 μL of a suspension of recombinant GST-Ral-RBD bound to glutathione beads (Sigma G4150) for 1 hour at 4°C with gentle rocking. After incubation, conjugates were pelleted by centrifugation, washed four times with ice-cold MAPK lysis buffer, and boiled in 2× Laemmli buffer. Suspensions were separated by SDS-PAGE on 15% acrylamide gels, and Western blot analysis was used to detect activated Rap1 (Santa Cruz Biotechnology antibody, 1:1,000 dilution) in the bound fractions and DAL-1 and 14-3-3 in the input fractions. Each experiment was done at least thrice with identical results.

Rho activation assay. Rho activation was determined using affinity chromatography methods identical to those described above for the Rap1 activation assay, except that activated Rho was precipitated with GST-Rhotekin bound to glutathione beads, and Western blots were probed with an anti-Rho antibody (Santa Cruz Biotechnology antibody, 1:500 dilution).

Transfections. IOMM-Lee cells and IOMM-Lee DAL-1-expressing stable cell lines were seeded in 100 mm dishes with complete DMEM plus 10% FCS at 300,000 per dish 18 hours before transfection. Transfections were done using the Lipotaxi transfection reagent (Stratagene, La Jolla, CA), and cells were harvested 48 hours posttransfection in NP40 lysis buffer after washing with ice-cold 1× TBS thrice. Protein concentrations in each lysate were determined by BCA assay (Pierce) before SDS-PAGE and Western blot analysis for specific proteins and phosphoproteins.

Small interfering RNA treatments. To achieve knockdown of Rac1 protein levels, IOMM-Lee cells were transiently transfected with SMARTpool small interfering RNAs (siRNA) directed against the Rac1 mRNA sequence (Upstate Biotechnologies). Briefly, cells were seeded in six-well plates, and were transfected at 50% to 70% confluency with 200 pmol Rac1 SMARTpool or negative control siRNA. The following day, cells were reseeded into 100 mm dishes, and grown for another 24 hours before harvesting in NP40 lysis buffer plus protease and phosphatase inhibitors.

Clonogenic assays. The effects of DAL-1 expression were analyzed using clonogenic assays by cotransfection of equimolar amounts of pcDNA3, pcDNA3.DAL-1, or pcDNA3.DAL-1ΔU2 in combination with MSCV, MSCV.$Rac1^{N17}$, or MSCV.$Rac1^{V12}$. A second set of clonogenic assays were done using pcDNA3, pcDNA3.DAL-1, and pcDNA3.$Rac1^{V12}$. Cells were seeded in six-well plates 18 hours before transfection at a density of 2×10^5 per well in complete DMEM, and were transfected with 2 pmol plasmid DNA using the Lipotaxi transfection reagent. Twenty-four hours post-transfection, cells were trypsinized and plated in 100 mm dishes at identical densities for each transfection. Transfected cells were selected in 500 μg/mL G418 for 10 to 14 days, and surviving colonies were counted. Transgene expression was confirmed for each experiment. Each experiment was done at least thrice with identical results.

Densitometric analysis. Western blots were scanned and analyzed using the Gel-Pro Analyzer 4.0 program (MediaCybernetics, Silver Spring, MD). Total density was measured for each sample, and fold differences were calculated using the density measured in the V3 sample as the baseline standard. At least three different Western blots were used to generate fold difference comparisons, and the values were averaged and then subjected to statistical analysis using the Student's *t* test to determine significance ($P < 0.05$).

Results and Discussion

Protein 4.1B/DAL-1 specifically activates a signaling cascade culminating in JNK activation. Previous studies from our laboratory have shown that protein 4.1B suppresses growth in human meningioma cells *in vitro* (16, 17). A fragment of the full-length protein 4.1B molecule, termed DAL-1, has been identified as the minimal region of protein 4.1B required to mediate protein 4.1B growth suppression. In these experiments, four independent methods, including clonogenic assays, direct cell counting, thymidine incorporation, and measures of apoptotic cell death, were used to show that protein 4.1B/DAL-1 expression resulted in reduced growth in several different 4.1B-deficient meningioma cell lines (16, 17, 20). Moreover, exogenous DAL-1 expression recapitulates the subcellular localization and protein-protein interactions observed with endogenous protein 4.1B. In a recent study, we identified the unique U2 region of protein 4.1B as a domain necessary for protein 4.1B/DAL-1 growth suppression in IOMM-Lee human meningioma cells (17). In that study, we showed that deletion of the U2 domain from the DAL-1 protein abolished its ability to suppress growth, and that expression of only this domain, when properly targeted to the plasma membrane, was sufficient to suppress growth in meningioma cells. In addition, we showed that expression of DAL-1 resulted in activation of JNK, and that this activation was dependent on expression of the U2 domain (17). These results suggested that protein 4.1B/DAL-1 growth regulation might require JNK pathway activation.

In an effort to define the intracellular signaling events leading to DAL-1-mediated JNK activation, we first wanted to confirm that the JNK activation we observed in IOMM-Lee human meningioma cells was unique to the JNK signaling cascade, or whether other critical growth regulatory pathways were similarly regulated by DAL-1. Lysates from two representative and independently isolated IOMM-Lee cell lines either expressing DAL-1 or empty vector were analyzed by Western blot for activated signaling molecules representing a number of different signaling pathways implicated in brain tumor growth regulation. These specific IOMM-Lee cell lines were chosen for three reasons. First, IOMM-Lee human meningioma cells lack endogenous protein 4.1B expression. Second, the levels of DAL-1 overexpression were similar to those observed in normal mouse brain (Supplementary Fig. S1). Third, we have previously used these same cell lines to show that DAL-1 expression results in reduced cell growth by thymidine incorporation (Supplementary Fig. S2), direct cell counting, and measures of cell death (17). We found that expression of DAL-1 specifically activated JNK in IOMM-Lee cells (Fig. 1*A*). No activation of Akt, S6K, S6, p42/p44-MAPK, or p38-MAPK (Supplementary Fig. S3) was observed, suggesting that protein 4.1B/DAL-1 uniquely activates JNK in meningioma cells.

To confirm that the JNK activation results obtained with the DAL-1 fragment reflected a property of the full-length 4.1B molecule, we transiently transfected IOMM-Lee cells with constructs containing either DAL-1 or full-length protein 4.1B. Lysates from these transfected cells were analyzed by Western blot for JNK activity using phosphospecific JNK antibodies (Fig. 1*B*). In contrast to IOMM-Lee cells transfected with empty vector, IOMM-Lee cells transfected with either DAL-1 or full-length protein 4.1B exhibited increased JNK activation, indicating that both protein 4.1B and its growth suppressor fragment, DAL-1, activate JNK. In addition, activation of JNK correlates with the growth suppressor function of DAL-1, as transfection of the inactive DAL-1 ΔU2 construct does not activate JNK (see below; Fig. 6*B*), consistent with our previous observations (17). Last, to provide a direct correlation between 4.1B expression, JNK activation, and cell growth, we used regulatable 4.1B-expressing IOMM-Lee cell lines. Upon the addition of doxycycline, protein 4.1B is expressed. Using one representative inducible 4.1B-expressing IOMM-Lee cell line, we observed decreased IOMM-Lee growth as measured by direct cell counting (Fig. 1*C*) and increased JNK activation (Fig. 1*C, inset*). Collectively, these results show that 4.1B/DAL-1 growth suppression correlates with the specific activation of JNK.

JNK activation is required for DAL-1 regulation of growth suppression. The mechanism(s) by which DAL-1 regulates cell

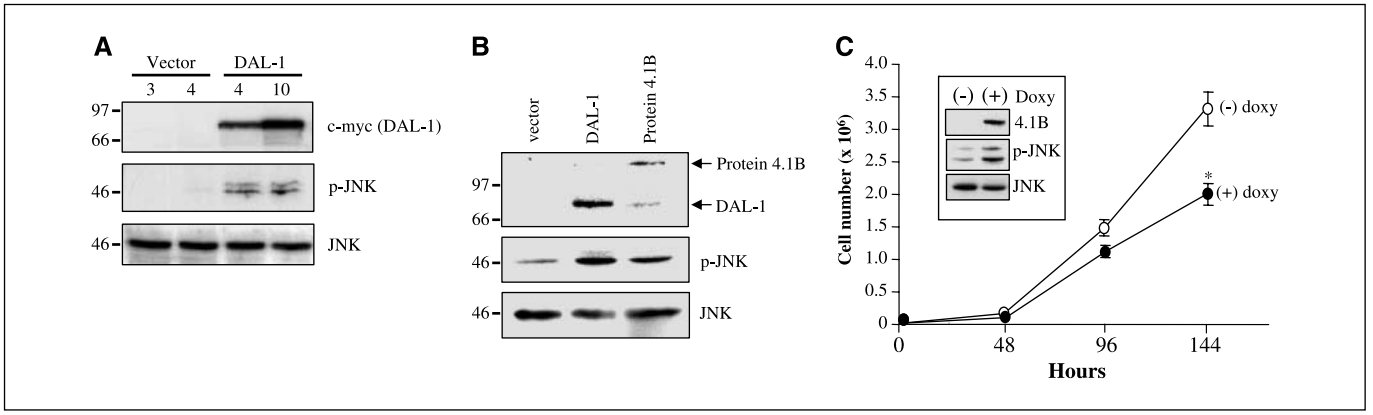

Figure 1. Expression of protein 4.1B/DAL-1 results in activation of the JNK pathway in IOMM-Lee meningioma cells. *A,* immunoblot analysis of two representative independently generated IOMM-Lee cells stably expressing DAL-1 (clones 4 and 10) revealed an increase in activated JNK (p-JNK) levels, compared with cells containing empty vector (clones 3 and 4). DAL-1 was expressed as a myc-tagged protein (detected with the 9E10 myc monoclonal antibody), and total JNK levels were used to control for equal protein loading. *B,* both DAL-1 and protein 4.1B expression resulted in JNK activation. Empty vector, DAL-1, and full-length protein 4.1B were transiently expressed in IOMM-Lee cells. Immunoblots of lysates showed that p-JNK levels increase when both DAL-1 and protein 4.1B were expressed. Total JNK expression was used an internal control for equal protein loading. *C,* JNK activation correlated with reduced proliferation resulting from protein 4.1B expression. IOMM-Lee cells were engineered to express full-length protein 4.1B upon treatment with doxycycline (*Doxy*). Cells were seeded at identical density (20,000 per well at time 0) in each well of a six-well plate, and were treated with doxycycline to induce protein 4.1B expression (●) or left untreated (○). Cell number was determined by direct cell counting at the time points shown along the *X* axis. Expression of protein 4.1B resulted in a significant decrease in cell proliferation. *, *P* value, Student's *t* test. Western blot (*inset*) showed a concomitant increase in activated JNK (p-JNK) upon 4.1B expression at 48 hours after the addition of doxycycline. Total JNK expression was used an internal control for equal protein loading.

growth remain poorly understood. Previous reports by our laboratory and others have implicated apoptosis as a possible mechanism underlying DAL-1 growth suppression (17, 21). Although apoptosis has been observed in cells expressing DAL-1 in more than one model system, it is important to note that the percentage of cells undergoing apoptosis in these cell populations is quite small. This observation suggested that additional mechanisms might be involved in protein 4.1B/DAL-1 regulation of cell proliferation.

JNK regulates cell growth and integrates signals from multiple stimuli involving both apoptotic and cell cycle control pathways (22–24), and has previously been identified as a regulator of Rb and E2F, which are important modulators of G_1-S transition during cell cycle progression (25, 26). Given that protein 4.1B/DAL-1 expression results in elevated levels of activated JNK (17), we next sought to determine whether molecules downstream of the Rb-E2F regulatory module were affected in IOMM-Lee cells expressing DAL-1. We initially focused our attention on the cyclin A protein because it is a well-described downstream target of the E2F transcription factor (27–30) and has been extensively studied as a marker for cell cycle progression (28–30).

Immunoblot analyses of lysates from IOMM-Lee cells stably expressing DAL-1 and the inactive DAL-1 ΔU2 fragment showed that cyclin A levels were markedly reduced only when the active DAL-1 molecule was expressed (Fig. 2*A*). Similar results were recently observed upon inducible protein 4.1B/DAL-1 expression in breast cancer cell lines (31). In these studies, protein 4.1B expression resulted in reduced cyclin A levels, decreased Rb hyperphosphorylation, and G_0-G_1 cell cycle growth arrest. In our experiments using IOMM-Lee meningioma cells, the effect of protein 4.1B/DAL-1 expression on cyclin A levels was dependent on expression of the U2 domain, as expression of the inactive ΔU2 DAL-1 mutant had no effect on cyclin A levels. Further, regulatable expression of protein 4.1B in IOMM-Lee cells results in reduced cyclin A levels, with a concomitant increase in JNK activation (p-JNK levels) as a function of time after the induction of 4.1B expression (Fig. 2*B*), indicating that the ability to regulate JNK activation and cyclin A protein levels is common to both DAL-1 and protein 4.1B.

We next examined the effect of DAL-1 expression on Rb phosphorylation based on previous studies linking JNK activation to Rb function (25, 26). We found that DAL-1 expression resulted in decreased Rb hyperphosphorylation in IOMM-Lee cells (Fig. 2*C*), and that this effect was also dependent on the presence of the U2 domain. These observations are consistent with recent findings on protein 4.1B/DAL-1 expression in breast cancer cell lines (31).

To provide a mechanistic link between DAL-1 expression, JNK activation, and cyclin A expression, we examined the consequence of JNK inhibition on DAL-1-mediated reduction in cyclin A

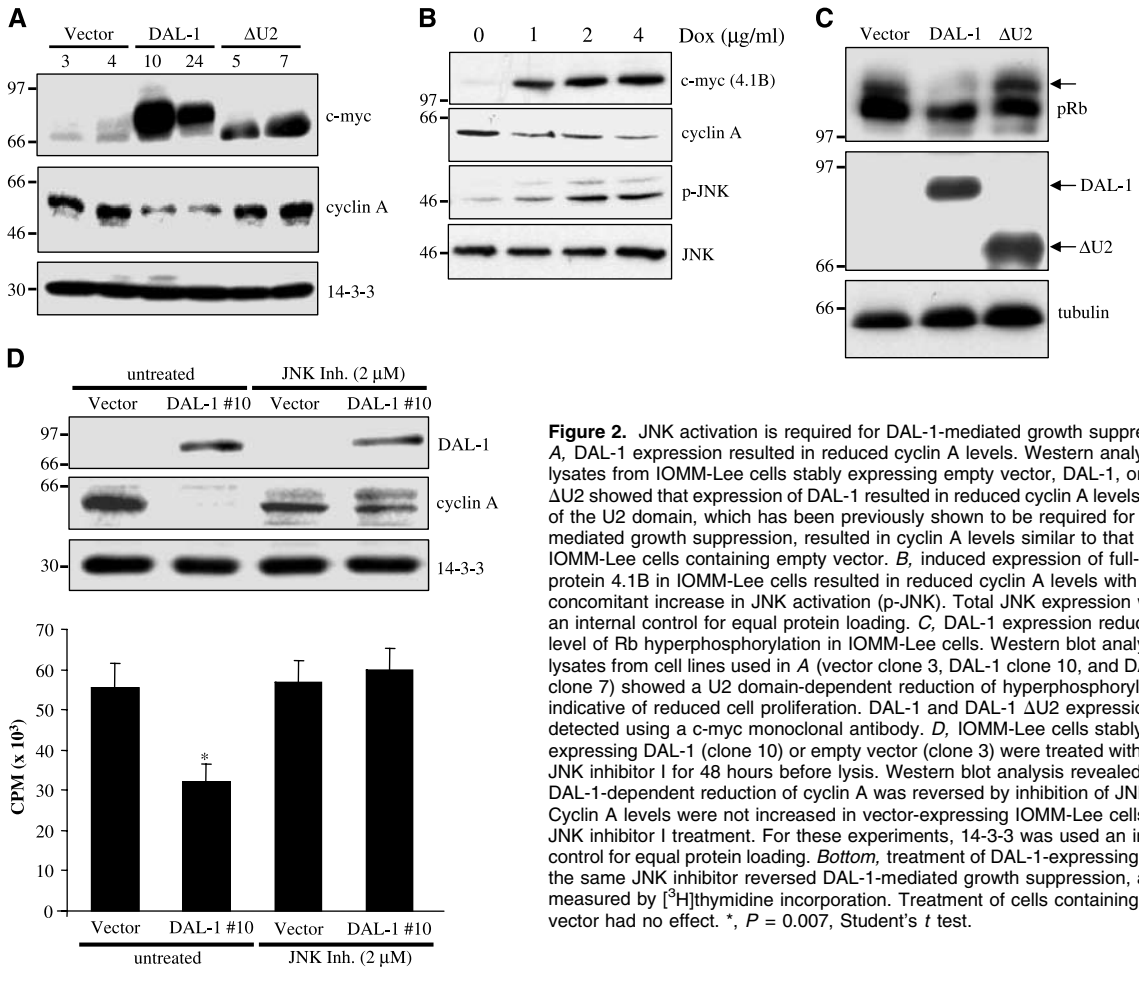

Figure 2. JNK activation is required for DAL-1-mediated growth suppression. *A,* DAL-1 expression resulted in reduced cyclin A levels. Western analyses of lysates from IOMM-Lee cells stably expressing empty vector, DAL-1, or DAL-1 ΔU2 showed that expression of DAL-1 resulted in reduced cyclin A levels. Deletion of the U2 domain, which has been previously shown to be required for DAL-1-mediated growth suppression, resulted in cyclin A levels similar to that seen in IOMM-Lee cells containing empty vector. *B,* induced expression of full-length protein 4.1B in IOMM-Lee cells resulted in reduced cyclin A levels with a concomitant increase in JNK activation (p-JNK). Total JNK expression was used an internal control for equal protein loading. *C,* DAL-1 expression reduced the level of Rb hyperphosphorylation in IOMM-Lee cells. Western blot analysis of lysates from cell lines used in *A* (vector clone 3, DAL-1 clone 10, and DAL-1 ΔU2 clone 7) showed a U2 domain-dependent reduction of hyperphosphorylated Rb, indicative of reduced cell proliferation. DAL-1 and DAL-1 ΔU2 expression was detected using a c-myc monoclonal antibody. *D,* IOMM-Lee cells stably expressing DAL-1 (clone 10) or empty vector (clone 3) were treated with 2 μmol/L JNK inhibitor I for 48 hours before lysis. Western blot analysis revealed that the DAL-1-dependent reduction of cyclin A was reversed by inhibition of JNK activity. Cyclin A levels were not increased in vector-expressing IOMM-Lee cells after JNK inhibitor I treatment. For these experiments, 14-3-3 was used an internal control for equal protein loading. *Bottom,* treatment of DAL-1-expressing cells with the same JNK inhibitor reversed DAL-1-mediated growth suppression, as measured by [^{3}H]thymidine incorporation. Treatment of cells containing empty vector had no effect. *, $P = 0.007$, Student's t test.

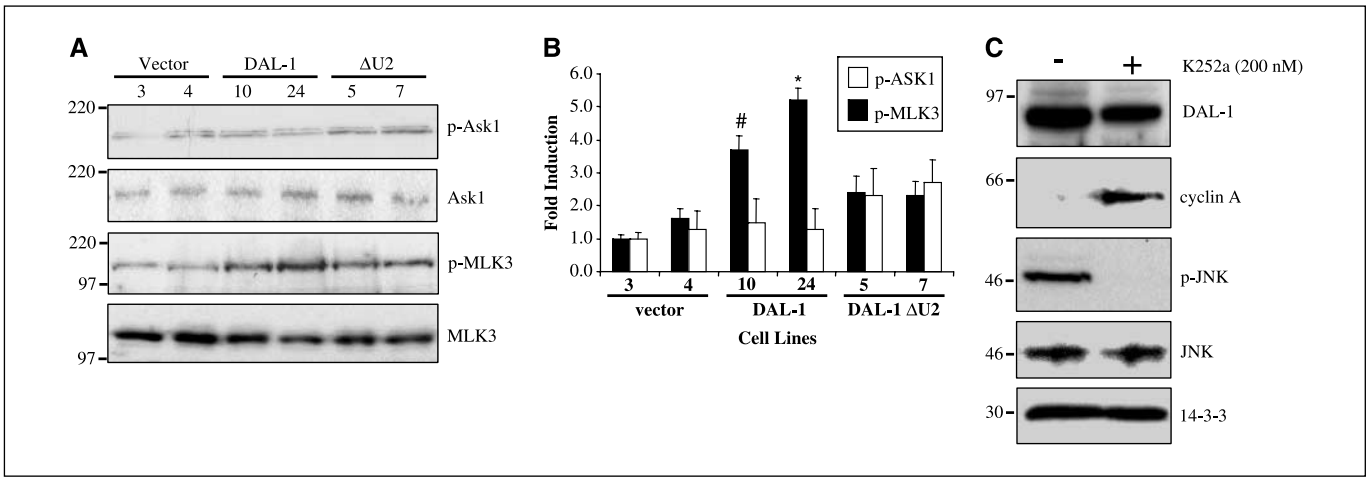

Figure 3. Activation of the mixed-lineage kinase, MLK3, is required for DAL-1 growth suppression. *A,* MLK3 was activated when active DAL-1, but not mutant DAL-1 ΔU2, was expressed. Western blot analysis was done on lysates from IOMM-Lee cells expressing empty vector (clones 3 and 4), DAL-1 (clones 10 and 24), or DAL-1 ΔU2 (clones 5 and 7). Phosphorylation of MLK3 was observed only when full-length DAL-1 was expressed. Phosphorylation of ASK1, another upstream activator of JNK, remained unchanged. *B,* densitometric quantitation from three independent experiments showed a 3.5- to 5.0-fold increase in MLK3 activation in DAL-1-expressing IOMM-Lee cells (clone 10) when normalized to total MLK3 expression. In contrast, ASK1 activation was unaffected by DAL-1 expression. Densitometric analysis was done using Gel Pro Analyzer. *, $P = 0.003$, Student's *t* test; #, $P = 0.023$. *C,* inhibition of MLK3 reversed the effect of DAL-1 expression on JNK activation and cellular proliferation. Cells expressing DAL-1 (clone 10) were treated with vehicle (DMSO) or the MLK3 inhibitor K252a (200 nmol/L) for 48 hours. Cell lysates were analyzed by Western blot for DAL-1, cyclin A, and p-JNK. Total JNK expression was used an internal control for equal protein loading. When cells were treated with the inhibitor, DAL-1-mediated JNK activation and cyclin A reduction was reversed.

expression and inhibition of cell proliferation. In these experiments, IOMM-Lee cells expressing vector or DAL-1 were treated with JNK Inhibitor I [(L)-HIV-TAT$_{48-57}$-PP-JBD$_{20}$, Calbiochem; refs. 32, 33]. A 48-hour treatment with a 2 μmol/L concentration of this inhibitor reversed the effect of DAL-1 on both cyclin A expression (Fig. 2*D, top*) and IOMM-Lee meningioma cell proliferation, as measured by [^{3}H]thymidine incorporation (Fig. 2*D, bottom*). The JNK inhibitors had no effect on protein 4.1B–deficient vector-containing IOMM-Lee cells. These findings show that JNK activation is required for DAL-1 regulation of cyclin A expression and cell proliferation.

MLK3 activation is required for DAL-1 activation of JNK and growth regulation. JNK is potently activated by a number of environmental signals, including growth factors, inflammatory cytokines, ceramides, and UV and γ radiation (22, 23). The core signaling unit for JNK activation involves the mixed-lineage protein kinases and the MAPK kinase kinase molecules (22, 23). Among these kinase molecules are MLK3 and ASK1 (34, 35).

To define the upstream signaling pathway leading to JNK activation, we examined the activation status of MLK3 and ASK1 in DAL-1-expressing IOMM-Lee cells. Western blot analyses of lysates from IOMM-Lee cells expressing DAL-1 showed a ~3.5- to 5.0-fold increase in MLK3 activation using phosphospecific antibodies (Fig. 3*A* and *B*). In contrast, activated ASK1 levels remained unchanged. Further, MLK3 activation was dependent on the presence of the U2 domain.

To show that MLK3 activation is required for DAL-1-mediated JNK activation and growth regulation, we treated DAL-1-expressing IOMM-Lee cells with an MLK3 inhibitor (K252a, Calbiochem; refs. 36, 37) and examined JNK activation and cyclin A protein expression. When cells expressing DAL-1 were treated with this inhibitor, the effects of DAL-1 expression on both JNK activation and cyclin A expression were reversed (Fig. 3*C*), indicating that DAL-1 regulation of JNK activation and cell growth, as reflected by cyclin A levels, requires MLK3 activation.

DAL-1 activation of JNK is dependent on Rac1 activation. Known activators of mixed-lineage kinases include members of the small Rho-family GTPases (34, 38–40). Among these GTPase molecules are the Rac, Rho, and cdc42 proteins. Given that previous studies have established a connection between JNK activation and Rac activation in numerous cell types and under a range of stimuli (22, 41–43), we investigated the potential role of Rac1 in the regulation of JNK by DAL-1. In these experiments, we showed that DAL-1 expression resulted in Rac1 activation using a Rac1 activity assay (Fig. 4*A, top*). GTPγS- and GDP-treated cell lysates were used as positive and negative controls for Rac1 activation, respectively. Vector controls showed low levels of basal Rac1 activity. As previously shown for JNK activation, the activation of Rac1 by DAL-1 was also dependent on the presence of the U2 domain (Fig. 4*A, bottom*).

To exclude the possibility that Rac1 activation represented a nonspecific effect of DAL-1 expression, we measured the activity of other related GTPase molecules, including Rap1, cdc42, and Rho, which are also activated by upstream regulators of Rac1 (e.g., Src family kinases; refs. 44, 45). In these experiments, expression of DAL-1 had no effect on the activity of any of these GTPase molecules (Fig. 4*B*). Collectively, these data suggest that DAL-1 specifically activates Rac1.

To provide direct evidence for a role for Rac1 in DAL-1-mediated activation of JNK and cell growth, we first introduced a dominant-negative form of the Rac1 molecule (Rac1^{N17}; ref. 43) into IOMM-Lee cells that stably expressed DAL-1. Introduction of Rac1^{N17} into DAL-1 expressing IOMM-Lee cells abolished JNK activation (Fig. 4*C*). Furthermore, the reduction in cyclin A expression, which results from DAL-1 expression, was also reversed upon the introduction of dominant-negative Rac1. Second, we reduced Rac1 levels in DAL-1-expressing cells by introducing specific siRNAs (Upstate Biotechnologies) to degrade Rac1 mRNA. Reduction of Rac1 protein levels using this strategy also attenuated DAL-1-mediated JNK activation (Fig. 4*D*). Collectively, these

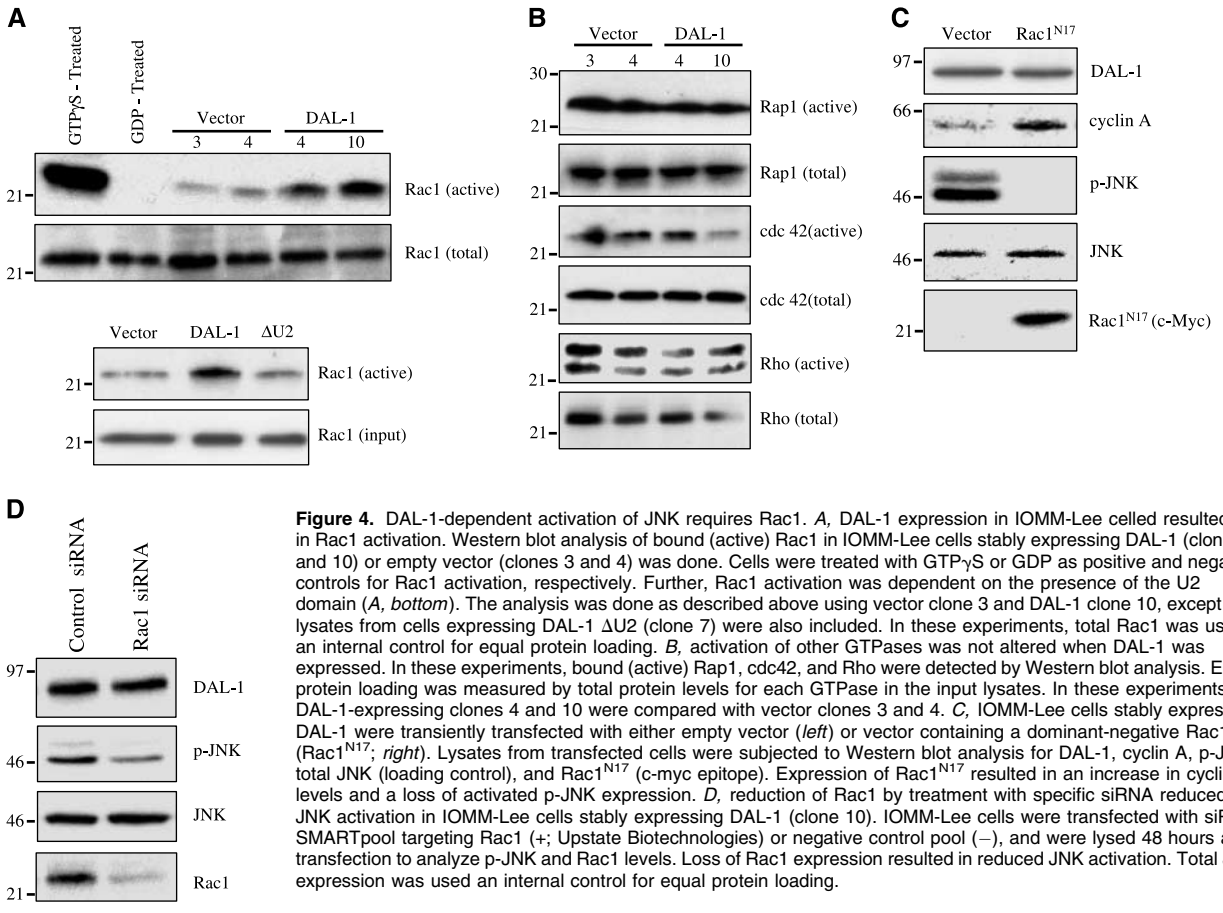

Figure 4. DAL-1-dependent activation of JNK requires Rac1. *A,* DAL-1 expression in IOMM-Lee celled resulted in Rac1 activation. Western blot analysis of bound (active) Rac1 in IOMM-Lee cells stably expressing DAL-1 (clones 4 and 10) or empty vector (clones 3 and 4) was done. Cells were treated with GTPγS or GDP as positive and negative controls for Rac1 activation, respectively. Further, Rac1 activation was dependent on the presence of the U2 domain (*A, bottom*). The analysis was done as described above using vector clone 3 and DAL-1 clone 10, except that lysates from cells expressing DAL-1 ΔU2 (clone 7) were also included. In these experiments, total Rac1 was used an internal control for equal protein loading. *B,* activation of other GTPases was not altered when DAL-1 was expressed. In these experiments, bound (active) Rap1, cdc42, and Rho were detected by Western blot analysis. Equal protein loading was measured by total protein levels for each GTPase in the input lysates. In these experiments, DAL-1-expressing clones 4 and 10 were compared with vector clones 3 and 4. *C,* IOMM-Lee cells stably expressing DAL-1 were transiently transfected with either empty vector (*left*) or vector containing a dominant-negative Rac1 ($Rac1^{N17}$; *right*). Lysates from transfected cells were subjected to Western blot analysis for DAL-1, cyclin A, p-JNK, total JNK (loading control), and $Rac1^{N17}$ (c-myc epitope). Expression of $Rac1^{N17}$ resulted in an increase in cyclin A levels and a loss of activated p-JNK expression. *D,* reduction of Rac1 by treatment with specific siRNA reduced JNK activation in IOMM-Lee cells stably expressing DAL-1 (clone 10). IOMM-Lee cells were transfected with siRNA SMARTpool targeting Rac1 (+; Upstate Biotechnologies) or negative control pool (−), and were lysed 48 hours after transfection to analyze p-JNK and Rac1 levels. Loss of Rac1 expression resulted in reduced JNK activation. Total JNK expression was used an internal control for equal protein loading.

results using two independent methods of silencing Rac1 activity strongly suggest that DAL-1-regulated JNK signaling requires Rac1 activation.

DAL-1–mediated growth suppression requires Rac1 activation. Given that the introduction of a dominant-negative Rac1 molecule into DAL-1-expressing IOMM-Lee meningioma cells was sufficient to abrogate DAL-1-mediated JNK activation, and that inhibition of JNK was sufficient to reverse the effect of DAL-1 on cyclin A protein levels, we next tested whether Rac1 hyperactivation was necessary for DAL-1 growth suppression. Clonogenic assays were used to determine the effect of dominant-negative Rac1 on the ability of DAL-1 to suppress growth in IOMM-Lee cells. As previously reported (10, 16, 17, 20), overexpression of DAL-1 results in a significant reduction in IOMM-Lee colony number when compared with vector-transfected controls (Fig. 5*A*). Cotransfection of a dominant-negative Rac1 ($Rac1^{N17}$) reduced the ability of DAL-1 expression to suppress colony formation. However, $Rac1^{N17}$ expression had no effect in IOMM-Lee cells expressing empty vector. Quantitation of these results from three independent data sets is shown in Fig. 5*B*. In contrast, expression of the DAL-1 ΔU2 mutant did not suppress colony formation relative to vector controls (quantitated data shown in Fig. 5*C*), and $Rac1^{N17}$ expression had no effect on cell number when coexpressed with the DAL-1 ΔU2 mutant.

In addition, we did experiments using a constitutively active Rac1 ($Rac1^{V12}$) mutant to determine whether activation of Rac1 could recapitulate DAL-1-mediated growth suppression. In contrast to the reversal of DAL-1-mediated growth suppression obtained in DAL-1-expressing IOMM-Lee cells upon $Rac1^{N17}$, expression of $Rac1^{V12}$ alone results in a similar reduction in IOMM-Lee colony number, when compared with DAL-1 expression (Fig. 5*D*). Further, we transfected DAL-1-expressing cells with $Rac1^{V12}$ and observed a 2.5-fold increase in JNK activation in DAL-1-expressing cells upon $Rac1^{V12}$ coexpression (Supplementary Fig. S4). Collectively, these data implicate Rac1 activation as a key mechanism underlying protein 4.1B/DAL-1–mediated growth suppression.

DAL-1–mediated activation of Rac1-JNK signaling requires Src activation. Several studies have implicated the Src oncoprotein as an upstream regulator of the Rac1-JNK signaling pathway (46, 47). These data, combined with our observations that Rac1-JNK signaling was activated by DAL-1, prompted us to investigate the possible role of the Src protein in DAL-1-mediated JNK signaling. Western blot analysis of IOMM-Lee cell lysates revealed that DAL-1 expression leads to activation of Src (Fig. 6*A*). Again, this activation is dependent on the presence of the U2 domain, as expression of the ΔU2 DAL-1 mutant did not result in Src activation. These observations were further supported by independently conducted transient transfection experiments that showed both Src and JNK activation upon DAL-1 expression (Fig. 6*B*).

Because previous studies have shown a requirement for Rac1 and JNK in Src regulated signaling (46, 47), we next sought to

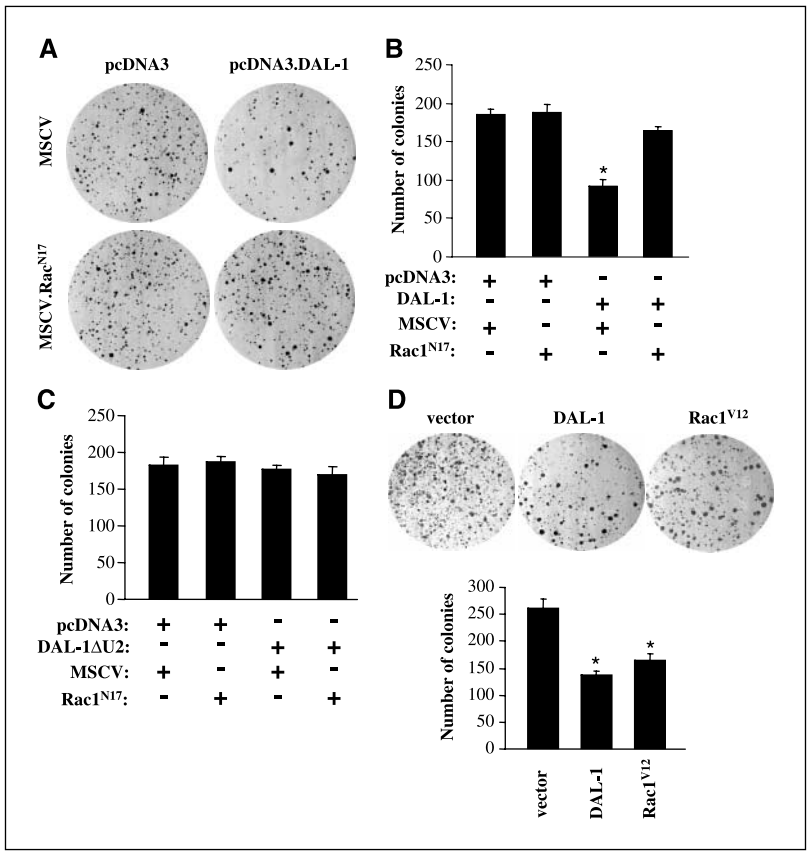

Figure 5. Rac1 is required for DAL-1-mediated growth suppression. *A,* clonogenic assays were used to show that Rac1^{N17} expression reversed the inhibitory effect of DAL-1 expression on cell growth. IOMM-Lee cells were cotransfected with empty vector (pcDNA3) or vector plus DAL-1 and empty MSCV or MSCV.Rac1^{N17} as indicated. After transfection, the cells were subjected to clonogenic selection in G418 for 10 to 14 days, and surviving colonies were stained with crystal violet and counted. A photograph of a representative dish from each cotransfection is shown. In these experiments, Rac1^{N17} expression reversed the growth suppression observed with DAL-1 expression. *B,* quantitative representation of the clonogenic assay results shown in *A*. Transfections were done thrice, and replicate plates from each transfection were counted to generate numbers for the analysis. *C,* experiments were done as in *A*. DAL-1 ΔU2 expression had no effect on colony formation, relative to empty vector, and cotransfection of Rac^{N17} with the DAL-1 ΔU2 mutant also had no effect on colony number. Results from three independent experiments were used for the quantitation. *D,* transfection with a constitutively active form of Rac1 (Rac1^{V12}) mimics DAL-1 growth suppression. Clonogenic assays were done on IOMM-Lee cells using empty pcDNA3, pcDNA3.DAL-1, or pcDNA3.Rac1^{V12}. Rac1^{V12} expression alone resulted in a similar reduction in colony formation compared with DAL-1 expression. *, $P < 0.003$, Student's *t* test. A photograph of a representative dish from each transfection is shown.

determine whether Src activation was required for DAL-1 modulation of Rac1-JNK signaling. We examined the levels of both JNK activation and Rac1 activation in DAL-1-expressing IOMM-Lee cells after treatment with multiple different Src inhibitors (herbimycin A, genestein, PP2, Calbiochem; refs. 48, 49). Inhibition of Src under conditions where DAL-1 is expressed resulted in a significant reduction in JNK activation (Fig. 6*C*), and a concomitant reduction in Rac1 activation (Fig. 6*D*). These results show that Src activation is necessary for DAL-1-mediated activation of Rac1-JNK signaling.

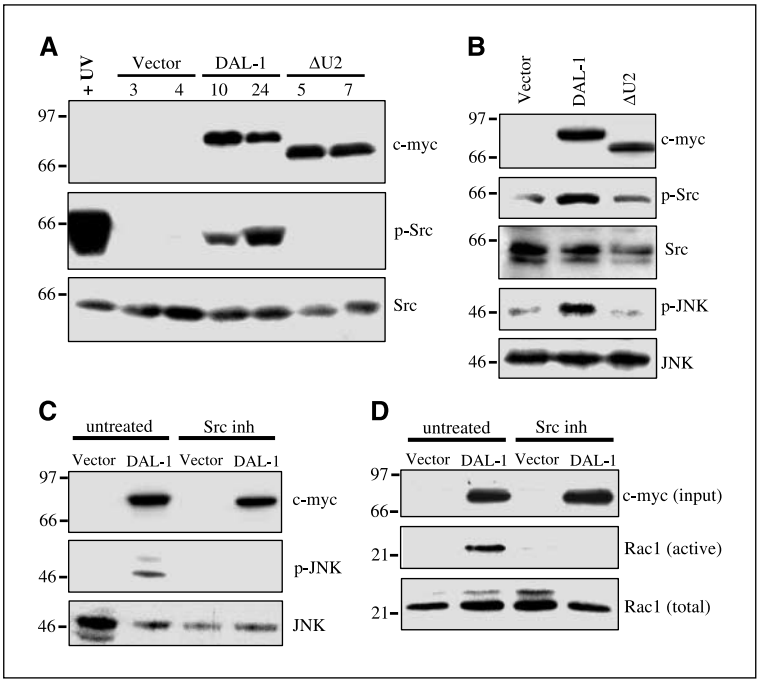

Figure 6. DAL-1-mediated activation of both JNK and Rac1 requires Src activation. *A,* Src was activated by DAL-1 expression. Western blot analysis of lysates from IOMM-Lee cells stably expressing vector (clones 3 and 4), DAL-1 (clones 10 and 24), or DAL-1 ΔU2 (clones 5 and 7) showed activation of Src (p-Src) upon DAL-1 expression. Src activation was dependent on the presence of the U2 domain. UV-treated cells were used as a positive control for Src activation. Total Src expression was used as an internal control for equal protein loading. *B,* IOMM-Lee cells were transiently transfected with empty vector, DAL-1, or the ΔU2 mutant. Western blot analysis of lysates revealed a U2-dependent increase in both activated p-Src and p-JNK expression. In these experiments, total JNK and total Src were used to control for equal protein loading. *C,* DAL-1-expressing IOMM-Lee cells (clone 10) and control vector IOMM-Lee cells (clone 3) were treated with Src inhibitors (5 μmol/L genistein plus 10 nmol/L PP2; Calbiochem) for 48 hours before harvest and lysis. As shown by Western blot, DAL-1-mediated JNK activation was eliminated by Src inhibitor treatment. In these experiments, control cells were treated with 10 nmol/L PP3 (Calbiochem). Total JNK expression was used an internal control for equal protein loading. *D,* IOMM-Lee cells expressing vector (clone 3) and DAL-1 (clone 10) were also treated with inhibitors as above, and lysates were analyzed for activated Rac1. DAL-1-dependent activation of Rac1 was abrogated when Src activation was inhibited. Total Rac1 expression was used an internal control for equal protein loading.

Protein 4.1B/DAL-1 growth regulation in meningioma. Given the dual role of protein 4.1B in regulating both cell proliferation (31) and apoptosis (21), we examined the activation status of JNK as a potential mechanism underlying protein 4.1B growth regulation. In this report, we show, for the first time, that protein 4.1B expression in meningioma cells results in JNK-mediated growth suppression by activating Src, Rac1, and MLK3. We also provide data to support a hierarchical sequence of signaling events using a combination of pharmacologic and genetic inhibitor studies, and establish a direct link between dysregulation of this pathway and protein 4.1B growth suppression.

One of the important unresolved questions centers on the precise mechanism of protein 4.1B activation of Src. Previous studies have shown that protein 4.1B/DAL-1 interacts with several protein partners, including 14-3-3 (50) and protein arginine *N*-methyltransferase-3 (PRMT-3; ref. 51). However, we have shown that protein 4.1B meningioma growth suppression does not require 14-3-3 binding (20). It is not known whether the interaction between protein 4.1B and PRMT3 is required for protein 4.1B growth suppression. Based on the fact that the region of protein 4.1B involved in PRMT3 binding maps outside the domains required for protein 4.1B/DAL-1 growth suppression, it is unlikely that PRMT binding is essential for protein 4.1B growth regulation in meningioma. Unfortunately, no binding partners have been identified that specifically associate with the critical U2 domain (17).

In an effort to determine how protein 4.1B/DAL-1 expression in meningioma cells results in Src activation, we examined the activation status of known positive regulators of Src. We found no evidence for a specific association between DAL-1 and Dock180, Tiam-1, Nck, Pyk2, growth factor receptor binding protein 2 or Vav-2, and no change in the activation status of Vav-2.[1] Moreover, we did not observe any effect of DAL-1 expression on receptor tyrosine kinase activation. Based on these results, we favor a mechanism in which protein 4.1B localization to the plasma membrane facilitates the formation of a signaling complex that culminates in the activation of Src. Experiments are currently in progress to identify the protein partners in this unique membrane signaling complex critical for protein 4.1B growth regulation in brain tumors.

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[1] M. Foster, M.A. Gerber, and D.H. Gutmann, unpublished observations.

References

1. Ruttledge MH, Sarrazin J, Rangaratnam S, et al. Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas. Nat Genet 1994;6:180–4.
2. Gutmann DH, Giordano MJ, Fishback AS, Guha A. Loss of merlin expression in sporadic meningiomas, ependymomas and schwannomas. Neurology 1997;49:267–70.
3. Huynh DP, Mautner V, Baser ME, Stavrou D, Pulst SM. Immunohistochemical detection of schwannomin and neurofibromin in vestibular schwannomas, ependymomas and meningiomas. J Neuropathol Exp Neurol 1997;56:382–90.
4. Leone PE, Bello MJ, de Campos JM, et al. NF2 gene mutations and allelic status of 1p, 14q and 22q in sporadic meningiomas. Oncogene 1999;18:2231–9.
5. Ueki K, Wen-Bin C, Narita Y, Asai A, Kirino T. Tight association of loss of merlin expression with loss of heterozygosity at chromosome 22q in sporadic meningiomas. Cancer Res 1999;59:5995–8.
6. Trofatter JA, MacCollin MM, Rutter JL, et al. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. Cell 1993;72:791–800.
7. Rouleau GA, Merel P, Lutchman M, et al. Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. Nature 1993;363:495–6.
8. Hoover KB, Bryant PJ. The genetics of the protein 4.1 family: organizers of the membrane and cytoskeleton. Curr Opin Cell Biol 2000;12:229–34.
9. Sun CX, Robb VA, Gutmann DH. Protein 4.1 tumor suppressors: getting a FERM grip on growth regulation. J Cell Sci 2002;115:3991–4000.
10. Tran YK, Bogler O, Gorse KM, Wieland I, Green MR, Newsham IF. A novel member of the NF2/ERM/4.1 superfamily with growth suppressing properties in lung cancer. Cancer Res 1999;59:35–43.
11. Singh PK, Gutmann DH, Fuller CE, Newsham IF, Perry A. Differential involvement of protein 4.1 family members DAL-1 and NF2 in intracranial and intraspinal ependymomas. Mod Pathol 2002;15:526–31.
12. Ohno N, Terada N, Murata S, et al. Immunolocalization of protein 4.1B/DAL-1 during neoplastic transformation of mouse and human intestinal epithelium. Histochem Cell Biol 2004;122:579–86.
13. Kikuchi S, Yamada D, Fukami T, et al. Promoter methylation of DAL-1/4.1B predicts poor prognosis in non-small cell lung cancer. Clin Cancer Res 2005;11:2954–61.
14. Gutmann DH, Donahoe J, Perry A, et al. Loss of DAL-1, a protein 4.1-related tumor suppressor, is an important early event in the pathogenesis of meningiomas. Hum Mol Genet 2000;9:1495–500.
15. Perry A, Cai DX, Scheithauer BW, et al. Merlin, DAL-1, and Progesterone receptor expression in clinicopathologic subsets of meningioma: a correlative immunohistochemical study of 175 cases. J Neuropathol Exp Neurol 2000;59:872–9.
16. Gutmann DH, Hirbe AC, Huang Z-y, Haipek CA. The protein 4.1 tumor suppressor, DAL-1, impairs cell motility, but regulates proliferation in a cell-type-specific fashion. Neurobiol Dis 2001;8:266–78.
17. Robb VA, Gerber MA, Hart-Mahon EK, Gutmann DH. Membrane localization of the U2 domain of protein 4.1B is necessary and sufficient for meningioma growth suppression. Oncogene 2005;24:1946–57.
18. Parra M, Gascard P, Walensky LD, et al. Molecular and Functional characterization of protein 4.1B, a novel member of the protein 4.1 family with high level, focal expression in brain. J Biol Chem 2000;275:3247–55.
19. Lee WH. Characterization of a newly established malignant meningioma cell line of the human brain: IOMM-Lee. Neurosurgery 1990;27:389–95.
20. Robb VA, Li W, Gutmann DH. Disruption of 14-3-3 binding does not impair protein 4.1B growth suppression. Oncogene 2004;23:3589–96.
21. Charboneau AL, Singh V, Yu T, Newsham IF. Suppression of growth and increased cellular attachment after expression of DAL-1 in MCF-7 breast cancer cells. Int J Cancer 2002;100:181–8.
22. Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Genet Dev 2002;12:14–21.
23. Manning AM, Davis RJ. Targeting JNK for therapeutic benefit: from junk to gold? Nat Rev Drug Discov 2003;2:554–65.
24. Kanda H, Masayuki M. Regulatory roles of JNK in programmed cell death. J Biochem 2004;136:1–6.
25. Wang S, Nath N, Minden A, Chellappan S. Regulation of Rb and E2F by signal transduction cascades: divergent effects of JNK1 and p38 kinases. EMBO J 1999;18:1559–70.
26. Chauhan D, Hideshima T, Treon S, et al. Functional interaction between retinoblastoma protein and stress-activated protein kinase in multiple myeloma cells. Cancer Res 1999;59:1192–5.
27. Schulze A, Zerfass K, Spitkovsky D, et al. Cell cycle regulation of the cyclin a gene promoter is mediated by a variant E2F site. Proc Natl Acad Sci U S A 1995;92:11264–8.
28. Rosenberg AR, Zindy F, Le Deist F, et al. Overexpression of human cyclin A advances entry into S phase. Oncogene 1995;10:1501–9.
29. Sherr CJ. Cancer cell cycles. Science 1996;274:1672–7.
30. Bukholm IR, Bukholm G, Nesland JM. Over-expression of cyclin A is highly associated with early relapse and reduced survival in patients with primary breast carcinomas. Int J Cancer 2001;93:283–7.
31. Kuns R, Kissil JL, Newsham IF, Jacks T, Gutmann DH, Sherman LS. Protein 4.1B expression is induced in mammary epithelial cells during pregnancy and regulates their proliferation. Oncogene 2005;24:6502–15.
32. Barr RK, Kendrick TS, Bogoyevitch MA. Identification of the critical features of a small peptide inhibitor of JNK activity. J Biol Chem 2002;277:10987–97.
33. Bonny C, Oberson A, Negri S, Sauser C, Schorderet DF. Cell-permeable peptide inhibitors of JNK: novel blockers of {β}-cell death. Diabetes 2001;50:77–82.
34. Teramoto H, Coso OA, Miyata H, Igishi T, Miki T, Gutkind JS. Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. J Biol Chem 1996;271:27225–8.
35. Tobiume K, Matsuzawa A, Takahashi T, et al. ASK1 is

required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Rep 2001;2:222–8.
36. Pan J, Zhang QG, Zhang GY. The neuroprotective effects of K252a through inhibiting MLK3/MKK7/JNK3 signaling pathway on ischemic brain injury in rat hippocampal CA1 region. Neuroscience 2005;131:147–59.
37. Roux PP, Dorval G, Boudreau M, et al. K252a and CEP1347 Are neuroprotective compounds that inhibit mixed-lineage kinase-3 and induce activation of Akt and ERK. J Biol Chem 2002;277:49473–80.
38. Bock BC, Vacratsis PO, Qamirani E, Gallo KA. Cdc-42-induced activation of the mixed-lineage kinase SPRK *in vivo*. J Biochem (Tokyo) 2000;275:14231–41.
39. Burbelo PD, Drechsel D, Hall A. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J Biochem (Tokyo) 1995; 270:29071–4.
40. Lambert JM, Karnoub AE, Graves LM, Campbell SL, Der CJ. Role of MLK3-mediated activation of p70 S6 kinase in Rac1 transformation. J Biol Chem 2002;277: 4770–7.
41. Rosso SB, Sussman D, Wynshaw-Boris A, Salinas PC. Wnt signaling through dishevelled, Rac and JNK regulates dendritic development. Nat Neurosci 2005;8:34–42.
42. Minden A, Lin A, Claret FX, Abo A, Karin M. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell 1995;81:1147–57.
43. Coso OA, Chiariello M, Yu JC, et al. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell 1995;81:1137–46.
44. Schmitt JM, Stork PJS. G(α) and G(β-γ) require distinct Src-dependent pathways to activate Rap1 and Ras. J Biol Chem 2002;277:43024–32.
45. Linseman DA, Hofmann F, Fisher SK. A role for the small molecular weight GTPases, Rho and Cdc42, in muscarinic receptor signaling to focal adhesion kinase. J Neurochem 2000;74:2010–20.
46. Turkson J, Bowman T, Adnane J, et al. Requirement for Ras/Rac1-mediated p38 and c-Jun N-terminal kinase signaling in Stat3 transcriptional activity induced by the Src oncoprotein. Mol Cell Biol 1999;19:7519–28.
47. Servitja J-M, Marinissen MJ, Sodhi A, Bustelo XR, Gutkind JS. Rac1 function is required for Src-induced transformation: evidence for a role of Tiam1 and Vav2 in Rac activation by Src. J Biol Chem 2003;278: 34339–46.
48. Hanke JH, Gardner JP, Dow RL, et al. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. J Biol Chem 1996;271:695–701.
49. Salazar EP, Rozengurt E. Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells. J Biol Chem 1999;274:28371–8.
50. Yu T, Robb VA, Singh V, Gutmann DH, Newsham IF. The 4.1/ezrin/radixin/moesin domain of the DAL-1/ Protein 4.1B tumour suppressor interacts with 14-3-3 proteins. Biochem J 2002;365:783–9.
51. Singh V, Miranda TB, Jiang W, et al. DAL-1/4.1B tumor suppressor interacts with protein arginine *N*-methyltransferase 3 (PRMT3) and inhibits its ability to methylate substrates *in vitro* and *in vivo*. Oncogene 2004;23:7761–71.