Epidermal Growth Factor Activates the Rho GTPase-activating Protein (GAP) Deleted in Liver Cancer 1 via Focal Adhesion Kinase and Protein Phosphatase 2A*

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Background: DLC1 is a RhoGAP tumor suppressor that inactivates Rho GTPases, but its link to growth factor regulation remains unknown.

Results: EGF activates the DLC1 phosphorylation-dephosphorylation cycle via MEK, FAK, and PP2A.

Conclusion: EGF regulates the spatiotemporal activation of DLC1 to control cell migration.

Significance: Signaling via the MEK/ERK-DLC1-FAK-PP2A quartet could integrate biochemical and mechanical cues to regulate cell dynamics.

Deleted in Liver Cancer 1 (DLC1) is a RHO GTPase-activating protein (GAP) that negatively regulates RHO. Through its GAP activity, it modulates the actin cytoskeleton network and focal adhesion dynamics, ultimately leading to suppression of cell invasion and metastasis. Despite its presence in various structural and signaling components, little is known about how the activity of DLC1 is regulated at focal adhesions. Here we show that EGF stimulation activates the GAP activity of DLC1 through a concerted mechanism involving DLC1 phosphorylation by MEK/ERK and its subsequent dephosphorylation by protein phosphatase 2A (PP2A) and inhibition of focal adhesion kinase by MEK/ERK to allow the binding between DLC1 and PP2A. Phosphoproteomics and mutation studies revealed that threonine 301 and serine 308 on DLC1, known previously to be mutated in certain cancers, are required for DLC1-PP2A interaction and the subsequent activation of DLC1 upon their dephosphorylation. The intricate interplay of this “MEK/ERK-focal adhesion kinase-DLC1-PP2A” quartet provides a novel checkpoint in the spatiotemporal control of cell spreading and cell motility.

Rho GTPases are key regulators of cell migration during normal tissue and organ development and in cancer progression. They affect the actin cytoskeleton through various downstream signaling pathways by cycling between the active GTP-bound and inactive GDP-bound forms. These are tightly regulated by their exchange factors (RhoGEFs) and GTPase-activating proteins (RhoGAPs), respectively (1). Cell migration involves complex but highly coordinated processes of cell-matrix interaction, cell protrusion, polarization, and retraction. A crucial step that determines the dynamics and function of cell-matrix interactions is the ability of cells to generate and resolve their focal adhesions (FAs) in response to physical and biochemical stimuli (2). Various core components for the FA machinery have been well described, which include focal adhesion kinase (FAK), integrin, talin, tensin, vinculin, and phosphatases, with more being continually identified (3). Despite the knowledge of the rich proteome of FAs, their regulation by Rho GTPases and the subsequent effect on cell migration and the cytoskeleton (4, 5), little is known about how these Rho GTPases are, in turn, regulated by the specific components of the “adhesome” in response to various progrowth and promigratory stimuli.

Of the approximately 70 RhoGAPs encoded by the human genome, only a few are reported to be localized at FAs, highlighting the selectivity in the regulation of Rho signaling by RhoGAP in this specific structure. Deleted in Liver Cancer 1 (DLC1) is one such RHOGAP. Initially identified to be deleted in liver cancer, loss of DLC1 expression, mainly via epigenetic silencing, has now been observed in prostate, lung, breast, colon, bladder, and head and neck cancers (6). DLC1 inhibits the activity of RHOA, RHOB, RHOC, and, to a lesser extent, Cdc42 (7, 8). It has also been reported to increase cell migration velocity but reduce its directionality (9). Furthermore, it inhibits cell invasion and metastasis in vivo (10) and is capable of inhibiting cell proliferation and promoting apoptosis (11). In addition to its RHOGAP domain, DLC1 contains the sterile alpha motif (SAM) and steroidogenic acute regulatory protein (StAR)-related lipid transfer protein modules and a unique serine-rich region (SRR). The START (12, 13), RHOGAP (14), SAM (15, 16) and SRR regions have been implicated in the regulation of cell morphology, migration, and tumor suppression.

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2 The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; FA, focal adhesion; FAK, Focal adhesion kinase; SRR, serine-rich region; MEF, mouse embryonic fibroblast; OA, okadaic acid; RE, R677E; SAM, sterile alpha motif; START, steroidogenic acute regulatory protein-related lipid transfer; RBD, Rho-binding domain; DPSS, diode-pumped solid state; PDMS, polydimethylsiloxane.

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Interaction of DLC1 with tensin proteins (17–19), talin, and FAK (20) is important for its optimal localization to the focal adhesion (21) and regulation of its RhoGAP activity (20). It has also been shown recently that DLC1-FAK interplay controls paxillin dynamics at focal adhesions during early cell spreading (22). While screening for potential mutational hotspots surrounding the focal adhesion-targeting and SRR of DLC1, two amino acid substitutions were identified, T301K and S308I, which reduced DLC1 RhoGAP activity (23). Furthermore, treatment with okadaic acid, the phosphatase PP2A inhibitor, has been shown to increase DLC1’s phosphorylation at Ser-327 and Ser-431, allowing its retention by 14-3-3 in the cytoplasm and leading to the loss of its RhoGAP activity (24). Additionally, B56, a regulatory subunit of PP2A, is known to be localized at focal adhesions (25), raising the possibility that PP2A could be functionally linked to FA dynamics. Taken together, it suggests that SRR of DLC1 may be a prime target of phosphorylation/dephosphorylation that could, in turn, regulate DLC1 functions. However, the trigger and mechanism of regulation of DLC1 RhoGAP activity and cellular functions by the phosphorylation/dephosphorylation circuitry at the FAs is still elusive. Here we report that EGF triggers DLC1 RhoGAP activation via a novel, two-step concerted mechanism. First, active MEK/ERK phosphorylates DLC1 and primes it for activation. Second, EGF stimulation inactivates FAK (26), leading to enhanced DLC1-PP2A interaction. Subsequent dephosphorylation of DLC1, in turn, activates its RhoGAP function, therefore providing an important temporal switch in FA-based motility.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—DLC1 was cloned into FLAG- and GFP-pXJ40 mammalian expression vectors (15). The truncation, deletion, and point mutants of DLC1 were generated using specific primers. Myc-PP2A<sub>C</sub> was a gift from Lin Sheng-Cai (Xiamen University, China), and the PP2A<sub>C</sub>-CS mutant was generated using site-directed mutagenesis. The FAK construct was a gift from Michael Sheetz (Columbia University) and was subcloned into the mCherry-pXJ40 vector. pGEX-Rhotekin-RBD (Rho-binding domain) was from S. Schoenwaelder (Monash University, Australia). Constructs were sequenced to confirm sequence fidelity.

**Cell Culture and Transfection**—HEK293T cells were grown in RPMI 1640 medium (HyClone) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 10 mM HEPES (HyClone). Cells at 60–80% confluence in 6-well plates were transfected with 1–2 μg of plasmid using Trans-IT LT1 (Mirus) according to the instructions of the manufacturer. HEK293T and HeLa JW cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 1 mM DTT and a mixture of protease inhibitors. Lysates were further diluted 10× with radiimmunoprecipitation assay lysis buffer and incubated with 20 μl of GST-tagged RBD bound to glutathione-Sepharose beads for 45 min at 4 °C. The beads were washed three times with lysis buffer, and bound proteins were resolved by SDS-PAGE for Western blot analyses.

**Cell Spreading Assay**—The transfected HeLa JW cells were starved for 18–24 h and subjected to various treatments. Post-treatment, the cells were trypsinized, resuspended in DMEM with 0% FBS, and recovered at 37 °C for 45 min. The samples were then diluted 5× and seeded onto 6-well plates that were pretreated with DMEM with 10% FBS overnight at 37 °C. The cells were allowed to spread, and the samples were fixed at various time points using 4% paraformaldehyde for 15–20 min at 37 °C, followed by three washes with 1 ml of PBS. The cells were imaged under a PerkinElmer Life sciences spinning disk confocal microscope with a ×10 objective using 488 DPSS (diode-pumped solid state) laser and bright field mode. Student’s t test and analysis of variance were used for statistical analysis (n = 160, p < 0.05).

**Wound Healing Assay**—PDMS (polydimethylsiloxane) strips were used to create a gap or a wound in the cultured cells, which were prepared using a Sylgard 184 silicone elastomer kit (Dow Corning, MI, USA). The silicone elastomer component was mixed with the curing agent in a ratio of 10:1 and degassed for 30 min after which it was poured into a 35 mm plate to a required depth. Subsequently, cross-linking of the elastomer was carried out at 80 °C for 2 h (28). The PDMS block was cut into strips of the required thickness and placed on 6-well plates (catalog no. BML-EI181, Enzo Life Sciences). FAK<sup>−/−</sup> MEFs and WT MEFs were cultured in DMEM supplemented with 4500 mg of glucose (Invitrogen), 10 mM sodium pyruvate (HyClone), and 10% (v/v) fetal bovine serum (Invitrogen). Electroporation of these cell lines was carried out using the Neon transfection system (Invitrogen). HeLa JW, FAK<sup>−/−</sup>, and MEFs were from Alexander Bershadsky (Weizmann Institute, Israel), Yasuhiro Sawada (Mechanobiology Institute, Singapore), and Michael Sheetz (Columbia University), respectively.

**Coimmunoprecipitation**—HEK293T and HeLa JW cells were lysed in 250 μl of radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl (pH 7.3), 150 mM NaCl, 0.75 mM EDTA, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, and freshly added protease inhibitors (Roche)) per 6-well plate. Aliquots were either directly analyzed by Western blotting or used for protein binding studies. For coimmunoprecipitation, lysates were incubated with anti-FLAG antibody conjugated to agarose beads (Sigma) at 4 °C for 4 h. The beads were washed with lysis buffer and analyzed by Western blotting. Primary antibodies were as follows: polyclonal anti-FLAG (Sigma), anti-RhoA (Sigma), anti-HA (Zymed Laboratories Inc.), monoclonal anti-phospho-ERK (Sigma), monoclonal anti-pan-ERK1/2 (BD Biosciences), polyclonal anti-α/β tubulin (Cell Signaling Technology), anti-α-myc (Zymed Laboratories Inc.), anti-DLC1 (BD Biosciences), monoclonal pan FAK (Abcam), anti-FAK Tyr(P)-397 (BD Biosciences), anti-FAK Ser(P)-910 (Abcam), and PP2A-C(α/β) (Santa Cruz Biotechnology).

**RhoGAP Activity Assay**—HeLa JW cells were lysed in radioimmune precipitation assay lysis buffer supplemented with 1 mM DTT and a mixture of protease inhibitors. Lysates were further diluted 10× with radiimmune precipitation assay lysis buffer and incubated with 20 μl of GST-tagged RBD bound to glutathione-Sepharose beads for 45 min at 4 °C. The beads were washed three times with lysis buffer, and bound proteins were resolved by SDS-PAGE for Western blot analyses.
before seeding of HeLa JW cells at high confluence. The cells were transfected, serum-starved, and subjected to various treatments. The PDMS strips were removed, creating a “wound” in the cell monolayer. The live imaging was done using an Olympus microscope with a ×10 objective at 37 °C with 5% CO₂. A warm white light-emitting diode was used for bright field imaging, and an Xcite Series 120Q fluorescence light source was used for fluorescence imaging with a FITC (U-MNIB) filter over a period of 50 h. Student’s t test and analysis of variance were used for statistical analysis (n = 4, p < 0.05).

**RESULTS**

**EGF Stimulates RhoGAP Activity and Phosphorylation of DLC1**—To investigate how the RhoGAP activity of DLC1 could be modulated by growth factor signaling, HeLa JW cells were transfected with WT or a RhoGAP activity-deficient mutant (R677E) of DLC1 (15), and the total level of active RHOA was then measured upon EGF stimulation of cells that were initially deprived of serum (Fig. 1A). The DLC1-R677E mutant is not capable of inactivating the active RHOA in the cells because of the mutation in the catalytic arginine finger, which is indispensable for its RhoGAP activity (9, 15, 29). As expected, WT but not the mutant DLC1 inactivated RHOA. However, this inactivation of RHOA by WT DLC1 occurred only upon EGF treatment. To test whether this effect of EGF occurred via phosphorylation of DLC1 and required activation of the canonical Ras/MEK/ERK module, FLAG-tagged DLC1 was analyzed and found to exhibit a lower electrophoretic mobility upon EGF stimulation that could be blocked by inhibiting MEK/ERK with U0126 (Fig. 1B). These results support the notion that EGF-mediated Ras/MEK/ERK signaling can activate DLC1, most likely by directly or indirectly phosphorylating it. With the knowledge that okadaic acid (OA), a Ser/Thr phosphatase inhibitor, helps maintain DLC1 in a phosphorylated state (24), we carried out an electrophoretic mobility shift assay on a denaturing gel in the presence of OA alone or together with EGF stimulation (Fig. 1C). The results showed that OA treatment alone did not result in the mobility shift and that the effect of OA probably occurred downstream of EGF stimulation.

To further confirm that EGF stimulation led to DLC1 phosphorylation, FLAG-tagged DLC1 was expressed and immunoprecipitated from unstimulated cells or cells stimulated with EGF with either OA or U0126 treatments (with appropriate controls). Peptides were then subjected to phosphoproteomic analysis to identify phosphorylated sites on DLC1. Several putative phosphorylation sites, unique to cells treated with both OA and EGF, were uncovered in the SRR of DLC1 (Fig. 1D). Other residues picked up in the phosphoproteomic analysis with EGF + U0126 and EGF + U0126 + OA treatments were eliminated as the putative phosphorylation sites to ensure that the sites identified were specifically those that resulted from the MEK/ERK pathway downstream of EGF stimulation. Using various truncation mutants of SRR, namely, SR1 (77–330), SR2 (331–485), and SR3 (486–652), it was further shown that only the SR1 fragment encompassing the putative sites 304–SSSSS–308 exhibited a mobility shift under EGF stimulation. Treatment with OA, on the other hand, did not lead to any mobility shift, indicating that DLC1 phosphorylation is primarily triggered by EGF-activated kinase and not merely induced by an inhibition of the phosphatase (Fig. 1E).

**MEK/ERK Promotes PP2A-DLC1 Interaction and Dephosphorylation of DLC1 to Activate Its RhoGAP Activity**—As in any phosphorylation/dephosphorylation circuitry of signaling control, phosphorylation of DLC1 should prime its interaction with a phosphatase to ensure its proper and timely regulation at the physiological level. To test this hypothesis and to investigate the interplay of kinase and phosphatase in mediating DLC1 function, we undertook the candidate approach and examined how PP2A would interact with DLC1 under EGF stimulation. Known to be associated with FAs (25), the effect of PP2A on DLC1 activity was shown through OA-mediated inhibition (24). Because interactions with phosphatases are highly transient, a catalytically dead mutant of PP2A, PP2AΔ–CS, was generated on the basis of the conserved cysteine residue (30, 31) to trap the PP2A-substrate complex. This mutant exhibited an acute binding to DLC1, peaking at 10 min after EGF stimulation, coinciding with maximal ERK activation (Fig. 2A), suggesting that PP2A binding to DLC1 is regulated by EGF signaling.

Next, on the basis of the potential target sites identified by the phosphoproteomics analyses, a deletion mutant, DLC1-ΔS (ΔS304–S308), was generated. Fig. 2B shows that, unlike the WT DLC1, DLC1-ΔS did not bind to PP2AΔ–CS even under EGF stimulation, implying that this region could serve directly as a PP2A binding site and/or indirectly as the target for PP2A-mediated dephosphorylation of DLC1. Residues Thr-301 and Ser-308 have been identified previously to be mutated in certain cancer patient samples (23). We next examined whether the phosphorylation status of these residues might have an impact on DLC1 binding to PP2A. To this end, dual phosphodefective (T301A/S308A) and phosphomimetic (T301D/S308D) mutants of DLC1 were generated, and binding studies were carried out. The result revealed that only the phosphomimetic mutant of DLC1 binds PP2A, whereas the phosphodefective mutant is incapable of binding to PP2A, even under EGF stimulation (Fig.
Similarly, binding of the WT or T301D/S308D DLC1 to PP2A<sub>C</sub>-CS was stimulated by constitutively active MEK2 (MEK2-SD) but not by kinase-dead MEK2 (MEK2-KA). However, even sustained MEK2 activation failed to promote T301A/S308A mutant binding to PP2A<sub>C</sub>-CS (Fig. 2D). These observations confirm that PP2A binding to DLC1 requires at least the Thr-301/Ser-308 motif and is downstream of EGF-mediated Mek2 activation.

To ensure that the stimulatory effect of EGF on the RhoGAP activity of DLC1 and DLC1 binding to PP2A are functionally linked and not because of independent events, HeLa JW cells expressing vector control, WT DLC1, DLC1-R677E, DLC1-
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T301A/S308A, or DLC1-T301D/S308D were stimulated with EGF (10 min), and lysates were prepared for the RhoGAP activity assay (Fig. 2E). Interestingly, the phosphodefective mutant form of DLC1 (T301A/S308A), but not the phosphomimetic form of DLC1, exhibited enhanced RhoGAP activity upon EGF stimulation. Taken together with the binding studies, these results suggest that DLC1 undergoes dephosphorylation, most likely at Thr-301 or/and Ser-308; that, most likely, the phosphorylation at these residues is important for PP2A recruitment to DLC1 because the phosphomimetic form, but not the phosphodefective form, of DLC1 binds to PP2A; and that the dephosphorylation event is necessary, but not sufficient on its own, to activate DLC1 because the phosphodefective mutant still requires EGF stimulation for its activation. Taking into consideration that these sites were identified using phosphoproteomics and found to be unique to EGF + OA-treated cells, PP2A is possibly responsible for their dephosphorylation. As such, other phosphorylation sites downstream of MEK/ERK, which is yet to be uncovered, are necessary to augment the PP2A-based activation regime.

EGF Stimulation Relieves FAK Inhibition of DLC1-PP2A Interaction—To examine whether DLC1-PP2A coupling would represent a universal mode of regulation for DLC1, we tested the binding of DLC1 with PP2AC-CS in HEK293T cells. Surprisingly, unlike the interaction in HeLa JW cells that showed absolute dependence on EGF stimulation, DLC1 and PP2AC-CS readily formed a complex in HEK293T cells despite the absence of any acute MEK/ERK activation (Fig. 3A), implying the existence of an inhibitory mechanism in HeLa JW cells that needs to be overcome by active MEK/ERK. To identify the molecular switch, we conducted an expression profile analysis of candidate proteins in HeLa JW and HEK293T cells and revealed that HeLa JW cells expressed abundant levels of constitutively active FAK, indicated by the detection of FAK Tyr(P)-397. In contrast, HEK293T cells expressed very little or no FAK at all (Fig. 3B). As separate internal controls, both cell lines were found to express similar levels of tubulin and the catalytic subunit of PP2A. We also observed that HeLa JW cells had lost the expression of DLC1. It has been shown previously that FAK binds to DLC1 and is important for its tumor-suppressive function (20). However, it is still not well understood how the FAK-DLC1 interaction would bring about such an effect. We therefore set out to determine whether FAK could influence the interaction between DLC1 and PP2AC-CS by comparing the binding of FLAG-tagged DLC1 and myc-tagged PP2AC-CS in control and FAK"−/−" MEFs. The results show that, with FAK being present, binding of DLC1 and PP2AC-CS required EGF stimulation (Fig. 3C). However, in FAK"−/−" MEFs, binding of DLC1 and PP2AC-CS was constitutive (Fig. 3D), reminiscent of that observed in FAK-deficient HEK293T cells. These observations suggest that FAK inhibits DLC1-PP2A interaction and that this inhibition is relieved upon MEK/ERK activation.

EGF Stimulation Controls the Timing of FAK Inhibition for Initiation of PP2A Binding to DLC1—How does EGF stimulation abrogate the inhibitory effect of FAK on DLC1-PP2A interaction? Previous studies have shown that EGF stimulation can lead to phosphorylation of FAK at Ser-910, priming it for subsequent Tyr-397 dephosphorylation (26) and inactivation. Treatment with U0126 confirmed that phosphorylation of Ser-910-FAK occurs downstream of MEK/ERK and that Ser(P)-910-FAK, in turn, leads to dephosphorylation of Tyr(P)-397-FAK (Fig. 4A). This also shows that, at 10 min, which corresponds to the maximal ERK activation by EGF in HeLa JW cells, there was an increase in Ser(P)-910-FAK and a simultaneous reduction in Tyr(P)-397-FAK. This profile of FAK inactivation is consistent with the maximal interaction of DLC1-PP2AC-CS observed under the same condition (Fig. 2A). To confirm the significance of FAK in regulating the coupling of DLC1-PP2AC-CS, further binding studies were repeated in HEK293T and HeLa JW cells, but now with FAK overexpression in HEK293T cells (Fig. 4B) and with treatment of a FAK inhibitor (PF-573228) to block FAK activation in FAK-expressing HeLa JW cells (Fig. 4C). Remarkably, the binding profiles of DLC1 and PP2AC-CS in HeLa JW and HEK293T cells were now reversed. The binding profile became EGF-dependent in HEK293T cells after introduction of FAK (Fig. 4B), whereas blocking FAK in HeLa JW made their binding constitutive and insensitive to EGF (Fig. 4C). Interestingly, the binding between DLC1 and FAK was reduced dramatically when the interaction between DLC1 and PP2A became the strongest after 10 min of EGF stimulation (Fig. 4B, right panel, compare the first and fifth rows). This profile suggests that DLC1-PP2A-FAK probably do not readily exist in a single trimeric complex but is consistent with a dynamic sequential order of binding between DLC1 with these two partners.

EGF Stimulation Orchestrates DLC1 Activity on RhoA by Exerting a Novel Two-step Control on FAK and PP2A—FAK has been shown to control RhoA activity in a tightly regulated manner (32). FAK"−/−" cells, in general, have elevated levels of active RhoA (33), which leads to impaired cell spreading and migration. To investigate how the interplay of DLC1, PP2A, and FAK could affect the RhoGAP activity of DLC1, inhibitors of FAK and PP2A were used on HeLa JW cells with and without DLC1 expression, and the corresponding level of active RHOA was measured after EGF stimulation. Fig. 5A shows that, in the

FIGURE 1. Phosphorylation of DLC1 by EGF triggers the activation of its GAP function toward RhoA. A–C and E, the experimental samples were analyzed using SDS-PAGE and Western blotting (WB). A, HeLa JW cells transfected with pXJ40-FLAG, FLAG-DLC1, and FLAG-DLC1-R677E were starved with serum-free medium for 24 h and then stimulated with 100 ng/ml of EGF for 10 min. Cell lysates were then subjected to RHOA activity analysis. The band intensity of the active RHOA and pan RHOA in each lane was measured, and the percentage of active RHOA was calculated. The readings were normalized to the vector-transfected cells. Vec, vector; PD, pulldown; WCL, whole cell lysate. B, HeLa JW cells were transfected (7C) with FLAG-DLC1 and starved for 24 h prior to treatment with U0126 (a MEK/ERK inhibitor), followed by 10 min of stimulation by 100 ng/ml EGF. C, HeLa JW cells were transfected with FLAG-DLC1 and starved for 24 h prior to treatment with OA, followed by 10 min of stimulation by 100 ng/ml EGF. D, phosphoproteomic analysis revealed residues in the serine-rich region of DLC1 that are potential phosphorylation sites downstream of OA treatment and EGF-stimulation. Conf, confidence levels. E, HeLa JW cells were transfected with various truncated constructs of the serine-rich region of DLC1 (FLAG-SR1, FLAG-SR2, and FLAG-SR3) and starved for 24 h prior to treatment with OA, followed by 10 min of stimulation by 100 ng/ml EGF. It is important to note that, because of the large molecular size of DLC1, 4% SDS-PAGE gel was used to observe the mobility shift. However, when analyzed on 10% SDS-PAGE gel as in all other experiments, such a mobility shift in full-length DLC1 became less apparent.
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absence of DLC1, active RHOA levels remained unperturbed despite treatment of cells with EGF and/or inhibitors of PP2A or FAK. However, when DLC1 was introduced into the cells, much of the RHOA became inactivated. RhoGAP activity was completely blocked when PP2A was inhibited by okadaic acid, whereas it remained intact on inhibition of FAK. Crucially, inhibition of FAK alone without EGF treatment failed to activate DLC1 despite DLC1 being overexpressed. Taken together,
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these and other results presented earlier highlight that DLC1 is the primary inactivator of RhoA under MEK/ERK activation. Active MEK/ERK executes two concerted pathways leading to DLC1 activation: first phosphorylation of DLC1 and inhibition of FAK and then promotion of PP2A to interact with and dephosphorylate DLC1 (Fig. 8).

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This unique two-step control of DLC1 by EGF stimulation provides an intricate temporal regulation of DLC1. This was evident in the RhoGAP activity assay comparing WT DLC1 and DLC1-H9004S, where the mutant lacking the region dephosphorylated by PP2A allowed its activation to occur at a time point earlier than WT DLC1 activation (Fig. 5B). The PP2A-mediated dephosphorylation event, therefore, acts as a critical checkpoint to prevent indiscriminate activation of DLC1 upon activation of the MEK/ERK pathway.

**Activation of DLC1 by MEK-FAK-PP2A interplay Enhances Cell Spreading but Retards Cell Migration**—The tumor-suppressive effects of DLC1, including its antimigratory and anti-metastatic functions, require its association with FAs (9, 10, 11).
17–20). However, the underlying molecular mechanism that activates DLC1 at the locale remains unknown. To elucidate how activation of DLC1 by the “MEK-FAK-PP2A signalome” could play a key role in these processes, we examined their effects on cell spreading and cell migration. Upon attachment to the extracellular matrix, cells begin to spread via RhoA inhibition, relieving the initial contractile forces and concomitant reciprocal activation of Rac and Cdc42 that drive membrane protrusion. Subsequently, focal complexes form and mature by reactivation of RhoA at the leading edge of cells to ensure persistent and directional migration (34).

Cells were transfected with vector control, WT DLC1, or its GAP-deficient mutant, R677E (RE), and a cell spreading assay was carried out as described under “Experimental Procedures.” The spread areas were calculated by drawing an outline of the cells using ImageJ software and measuring the area enclosed in the outline. A, the graphs were plotted as spread areas over time for pXJ40, DLC1, and DLC1-R677E. B, the spread area 60 min post-plating was used for comparison of various treatments. The different letters are indicative of statistical significance with respect to each other, and the error bars denote mean ± S.E. Analysis of variance was used for statistical analysis (n = 160, p < 0.05). Vec, vector. C, representative data showing spread cells with and without EGF stimulation at 60 min.
transfected cells exhibited greater spreading (determined by cell spread area) compared with the vector or RE-transfected cells. FAK inhibition has been shown to increase active RhoA in the cell (32) and reduce the cell spread area (35). As expected, treatment with a FAK inhibitor appeared to reduce the spread area in DLC1-transfected cells, but this effect was abrogated upon EGF treatment (Fig. 6B). These results are consistent with the notion that RHOA was inactivated by DLC1 via a GAP-dependent process that is blocked by FAK but reversible by EGF stimulation, as seen in the RhoGAP activity assay shown in Fig. 5A.

In comparison, cells transfected with the phosphodeficient mutant, T301A/S308A, which mimics the dephosphorylated form of DLC1, demonstrated a higher spreading area when compared with the phosphomimetic mutant, T301D/S308D, which mimics the MEK/ERK-mediated and constitutively phosphorylated form of DLC1 (Fig. 6B). Most significantly, EGF stimulation enhanced cell spreading elicited by T301A/S308A, irrespective of FAK inhibition, because MEK/ERK activation by EGF had led to FAK inhibition, as shown earlier. In contrast, EGF treatment only marginally enhanced cell spreading in T301D/S308D-transfected cells, consistent with the failure of T301D/S308D cells to inactivate RHOA, despite stimulation of cells with EGF, as seen in the RhoGAP activity assay (Fig. 2D). Fig. 6C is a collage of representative spread cells with and without EGF stimulation.

DLC1 has been shown previously to impair wound healing because of a reduction in cell directionality (9). To extend the significance of this novel regulatory loop to cell migration, cells were transfected with vector control, DLC1, or DLC1-RE, and their ability to migrate through an open space was monitored in the presence or absence of EGF stimulation over 50 h, a period that is required for the slowly migrating HeLa cells. Although this duration has been shown previously to be sufficient for the purpose of complete wound closure, the use of low-serum medium under our experimental conditions would slow down the rate of wound closure. Although the expression of DLC1 alone did not affect cell migration, treatment with EGF stimulated the potency of DLC1 to retard wound closure in a process that was further augmented by FAK inhibition. In contrast, although RE alone appeared to reduce cell migration by 25% because of a GAP-independent process, as reported previously (9), EGF stimulation failed to enhance the effect of RE, confirming that the impact of EGF-DLC1 on retarding cell migration was essentially due to the stimulation of the RhoGAP activity of DLC1. Unexpectedly, inhibiting FAK in cells expressing RE led to greatly enhanced cell migration, most likely because of cross-talk between FAK inhibition and an as yet unidentified GAP-independent mechanism (Fig. 7, A and B).

All of these results suggest a novel signaling circuitry that is centered in FAs, whereby EGF-mediated MEK/ERK activation leads to FAK inhibition that would otherwise suppress the ability of PP2A to interact with DLC1. Consequently, dephosphorylation of DLC1 by PP2A stimulates its RhoGAP activity, allowing it to mitigate its various downstream effects on cellular dynamics.

DISCUSSION

The tumor suppressor role of DLC1 requires its RhoGAP domain as well as its other regions to act via multiple mechanisms (9). Various processes modulate the functions of DLC1: phosphorylation by Akt (36), interactions with FAK and the tensin family proteins targeting it to FAs (17, 20, 37), its dimerization (38), or masking by other protein partners (24, 39). Despite extensive information regarding the functional regulation of DLC1, very little is known about how the regulation and GAP function of DLC1 are coupled to growth-promoting signals and how these are specifically linked to FA dynamics. It has been shown that, upon EGF stimulation, there is an expression switch from tensin3 to cten, leading to a drastic increase in active RHOA along the edges of a cell undergoing random migration. This effect of the tensin3/cten switch has been believed to be mediated via their ability to interact with DLC1 and the inability to activate it (16). The activation of ERK, however, was seen to have no apparent effect on DLC1 at the transcript levels. Moreover, no correlation was shown with respect to the protein levels or the phosphorylation status of DLC1.

Deregulation of the Ras/MAPK pathway is a major factor in cancer development. However, cancer is a multifactorial disease that can also result from the inactivation of a tumor suppressor. Here we show that activation of MEK/ERK by EGF stimulation not only leads to phosphorylation of DLC1 but that it also releases the inhibitory effect of FAK that would otherwise suppress the ability of PP2A to dephosphorylate and activate DLC1 (Fig. 8). The putative PP2A target sites identified on DLC1, Thr-301 and Ser-308, are known to be mutated in certain cancers (23) and are shown here as indispensable to the DLC1-PP2A interaction. It is therefore possible that, under oncogenic EGF signaling, the loss of PP2A action on DLC1 because of mutations would render the tumor suppressor DLC1 inactive and incapable of suppressing cell migration while promoting tumor growth.

Rho GTPases and their regulators, GEFs and GAPs, play a central role in controlling cell dynamics through their action on the actin cytoskeleton network, especially in response to integrin-mediated mechanotransduction (40). FAK associates with integrins and responds to mechanical stimuli by undergoing a conformational change, leading to its activation (41, 42). FAK has been shown to phosphorylate p190RhoGEF and p190RhoGAP to activate them. Activation of these molecules occurs independently at different stages of cell spreading, therefore regulating RhoGTPases in a spatiotemporal manner (32). Aberrant regulation of Rho GTPases is often related to cancer progression, and an increase in p190RhoGAP activity is observed in cancers where FAK is found to be up-regulated, leading to increased RhoA activity in the cells (41). In this study, we show that the presence of FAK suppresses DLC1 activation, which correlates to higher levels of RhoA activity. FAK expression and signaling is elevated in highly malignant cancers, which could be a result of not only activating a RhoGEF (41) but also inactivating a RhoGAP (in this case, DLC1). Because DLC1 and FAK work in concert to regulate paxillin dynamics in early cell spreading (22), this FAK-DLC1-PP2A network could provide

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an important spatiotemporal switch to regulate the dynamics of focal adhesions.

Function of FAK is also linked to ERK signaling. It has been shown that ERK-mediated phosphorylation of FAK at Ser-910 primes it for inactivation, which leads to the disassembly and increased turnover of focal adhesions (26). Apart from this, ERK is also involved in recruiting FAK to new focal adhesions, albeit indirectly via paxillin phosphorylation (41). Activation of the Ras/MAPK pathway downstream of growth factor stimulation requires the adaptor protein GRB2 (43). Interestingly, phosphorylation of Tyr-925 on FAK by Src provides the docking site for GRB2 (44), starting off the signaling cascade. As shown here, ERK phosphorylation is responsible for the activation of DLC1. Also, it is known that DLC1 overexpression leads to the dephosphorylation of Tyr(P)-925-FAK (9), raising the possibility of DLC1 controlling its own activation via a feedback loop.

**FIGURE 7.** DLC1-FAK-PP2A interplay retards cell migration in response to MEK/ERK. **A**, HeLa JW cells transfected with GFP-vector, GFP-DLC1, and GFP-DLC1-R677E were starved for 18–24 h in serum-free medium and then treated with FAK inhibitor and 100 ng/ml EGF. The area covered by the cells during migration was calculated using ImageJ software. Analysis of variance was used for statistical analysis (n = 4; *, p < 0.05). Error bars denote mean ± S.E. VEC, vector. B, representative data of the images obtained for the wound healing assay.
action occurs only upon inactivation of FAK and its dissociation from DLC1. This observation raises the question of how FAK inhibits their binding. Does FAK inhibition allow for DLC1-FAK decoupling, which, in turn, mediates PP2A interaction? Or does the inhibition of FAK affect its functions as a kinase, preventing it from phosphorylating DLC1 or PP2A, which, in turn, allows for the binding? Although the underlying molecular mechanisms for FAK-mediated inhibition of DLC1 and PP2A interaction remain to be investigated, our findings highlight the emergence of a cross-talk between biochemical signals downstream of EGFR/MEK/ERK and the mechanosensing module of FAK/RHO, modulated via the action of DLC1 RHOGAP and PP2A (Fig. 8). The intricate interplay of this novel MEK-FAK-DLC1-PP2A quartet provides an important spatiotemporal switch and defines a novel signaling mechanism that could integrate both biochemical and mechanical stimuli in cell motility and tissue/organ growth and to provide potential targets for future therapeutic intervention in cancer development.

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