Role of DLC-1, a Tumor Suppressor Protein with RhoGAP activity, in Regulation of the Cytoskeleton and Cell Motility

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I. Overview

DLC-1 was originally identified as a potential tumor suppressor. One of the key biochemical functions of DLC-1 is to serve as a GTPase activating protein (GAP) for members of the Rho family of GTPases, particularly Rho A-C and CDC 42. Since these GTPases are critically involved in regulation of the cytoskeleton and cell migration, it seems clear that DLC-1 will also influence these processes. In this review we examine basic aspects of the actin cyoskeleton and how it relates to cell motility. We then delineate the characteristics of DLC-1 and other members of its family, and describe how they may have multiple effects on the regulation of cell polarity, actin organization, and cell migration.

II. Basic Aspects of Cytoskeletal Function in Cell Migration and Tumor Cell Invasion

Cell Migration and Tumor Invasion

The invasive and metastatic characteristics of cancer cells are dependent on their ability to migrate effectively through the tumor microenvironment. Thus alteration in migratory ability is one of the hallmarks of the epithelial-mesenchymal transition that is so pivotal to tumor progression [1]. Cell migration is an extraordinarily complex process involving many different structural proteins and signaling pathways [2], and it is manifested differently in various cell types and under differing environmental conditions. The classic model of cell migration pertains to fibroblast-type cells migrating on a two-dimensional surface and is typified by cell polarization with an extensive leading lamellipodium and a narrow retracting tail. However, many other morphologies of cell locomotion can occur such as the pseudopodial-type migration associated with hematopoietic cells and the movements of epithelial sheets without loss of cell-cell contact [3]. Further, it is becoming increasing clear that cells migrate very differently in a three-dimensional tissue or culture environment than they do in two-dimensional situations. Cell motility is intimately linked to the functions of the actinomyosin cytoskeleton and involves iterative and cyclical regulation of key aspects of cytoskeletal function [4,5] Rho family small GTPases are key regulators of signaling pathways that regulate actin organization and cell migration [6,7]. In the sections below we will describe some of the major pathways that control cell polarity and the functions of the actin-myosin contractile apparatus. In particular, we focus on the function of DLC-1, a negative regulator of Rho GTPase function [8]. We discuss how the loss of DLC-1 may lead to aberrant Rho GTPase function and contribute to the aberrant migration, invasion and metastatic properties of cancer cells.

Cell Matrix Interaction and Cell Motility

In order for cells to move they must grip and pull against the extracellular matrix (ECM). Although other proteins and glycosaminoglycans also play a part, it is the integrin family of heterodimeric transmembrane proteins that plays the primary role in mechanically linking

between the multiple proteins of the ECM and the actin cytoskeleton [9]. Various integrins have extracellular domains with affinities for different ECM proteins such as fibronectin, laminin or collagen, while the intracellular tails of integrins can link directly to specialized cytoskeletal proteins including talin, filamin and paxillin that then directly or indirectly bind actin, thus providing a bridge between the exterior and interior of the cell. The specialized organelles of integrin-mediated cell-matrix adhesions are called focal contacts or focal adhesions and are complex, dynamic multiprotein structures [10,11]. Although integrins are key structural molecules it is important to realize that they also are vitally important in a number of signal transduction processes. Thus our laboratory and others have extensively explored the impact of integrins on the ERK mitogen-activated protein kinase pathway, as well as on other key signaling processes that impact on cell proliferation or differentiation [12,13]. It is also clear that integrin signaling plays a major role in the regulation of directional cell migration [14].

Role of Rho Family Proteins in Cytoskeletal Regulation

Starting with the seminal observations of Hall and colleagues in the early 1990s [15], it has become manifest that Rho family GTPases are critically involved in extracellular signal-stimulated regulation of the cytoskeleton and cell motility. The family is comprised of 20 mammalian members, with RhoA, Rac1 and Cdc42 the most studied and best characterized [16]. Rho GTPases function as GDP/GTP-regulated binary switches (Fig. 1). Activated cell surface receptors and integrins stimulate guanine nucleotide exchange factors (RhoGEFs) to promote formation of the active GTP-bound protein, which then engages downstream effectors. GTPase activating proteins (RhoGAPs) stimulate GTP hydrolysis, returning the GTPase to the inactive GDP-bound state. Less is known regarding how RhoGAPs may be regulated by extracellular signals.

Rho GTPases regulate distinct actin reorganization processes. A very simplistic summary would indicate that active RhoA promotes actin filament bundling (stress fibers) and focal adhesion formation, active Rac1 promotes actin accumulation at the leading edge of migrating cells and the extension of the lamellipodium, and active Cdc42 controls cell polarity and extension of actin microspikes and filopodia. Obviously the true situation is much more complex [17] but the simple picture will be helpful to keep in mind in the more detailed discussion below. An exciting new aspect of Rho and other small GTPases is the visualization of the active states of these GTPases in living cells, clearly demonstrating that extracellular stimuli cause a precise spatio-temporal activation of the GTPase that is intimately associated with the formation of actin structures and the directionality of migration [18,19].

Basic aspects of actinomyosin network organization

The lamellipodium, a key structure underlying cell motility, is supported by a highly branched actin network. Lateral branching of actin is mediated by the binding of the multi-protein Arp2,3 complex to an existing actin filament and is regulated by a set of proteins that includes Wave/ Scar, Abi,Nap125, Sra-1 and HSPC-300 that in turn is regulated by the Rac GTPase; alternatively the Arp2,3 complex can be regulated by WASP or N-WASP which are activated via Cdc42 [20,21]. Another key aspect of lamellipodial function, essential to cell migration, concerns the actin severing activity of cofilin and related proteins. Cofilin's severing ability is inactivated by phosphorylation at the serine 3 position, a process controlled by the action of kinases, including LIM kinase and TES kinases, and of phosphatases, particularly of the slingshot and chronophin families [22,23]. Regulation of this phosphorylation process can in part be traced back to Rho family GTPases; for example Rac or Cdc42-mediated activation of PAK kinases leads to phosphorylation and activation of LIM kinase thus increasing cofilin activity [24]. An interesting recent finding is the role of the coronin 1b protein as a coordinator of the activities of cofilin and the Arp2/3 complex in lamellipodial regulation [25]. The VASP/

Ena proteins represent another important set of modulators of lamellipodial function. These proteins inhibit capping of actin filament barbed ends and also decrease the density of Arp2/3 mediated branching; this serves to regulate the overall geometry of the lamellipodium [26]. The VASP/Ena proteins are regulated in part via phosphorylation by cyclic AMP and cyclic GMP activated kinases.

Contractility is a key aspect of cell shape and migration. Rho A contributes to contractility through its ability to trigger increased phosphorylation of the light chain of myosin II. Rho activates Rho kinases (ROCK I and II) which can both directly phosphorylate myosin light chain and also phosphorylate and inhibit myosin light chain phosphatase, both events leading to increased myosin light chain phosphorylation, increased actinomyosin contractility, and the enhanced formation of stress fibers [17,27,28]. In addition Rho also contributes to stress fiber formation by activating the formin protein mDia1 that, in cooperation with profilin, promotes barbed-end growth of long actin filaments [29].

Regulation of Polarity and Directionality

Migrating cells assume a polarized form with a leading lamellipodium and a retracting tail. This polarity is essential for maintaining directional rather than random cell migration. Rac, and especially Cdc42, play essential roles in initiating and maintaining cell polarity, acting through several different mechanisms. One important aspect involves the formation of a complex between PAK kinase, the Rac-specific GEF PIX, the Arf small GTPase-specific GAP Git1 and the focal adhesion protein paxillin that contributes to localized activation of Rac near the leading edge; in contrast, formation of a complex of comprised only of paxillin and Git1 in the tail leads to Rac inactivation [30,31]. Another key aspect of cell polarity involves Cdc42 regulation of the Par3/Par6/atypical PKC complex that is involved in microtubule function and leads to orientation of the microtubule organizing center in the direction of the leading edge [32]. As mentioned above Cdc42 also contributes to formation of highly polarized filopodia, acting in part via the formin mDia2 to promote extension at the barbed end of the actin filament [3]. Some of the roles of Rho GTPases in regulation of cell polarity and actin organization (and thus possible sites of DLC-1 actions) are highlighted in Fig 2.

Endo/Exocytosis and Cell Migration

It has long been thought that vesicle trafficking processes contribute to cell motility, for example by bringing adhesion receptors to the leading edge of the cell [4]. However, recent studies have unveiled complex interactions between the Rho, Arf and Rab families of small G-proteins that link vesicular trafficking to cell migration via multiple pathways. The Arf proteins promote vesicle formation by assisting in the assembly of coat-protein complexes [33]. In particular, Arf6 operates in the cell periphery and endosomes where it can recruit and activate phosphatidylinositol kinases. There is also a complicated relationship between Arf6 activation and Rac activation; this may be mediated in part through the Dock180-Elmo complex which is a Rac-specific GEF. In addition, Rac associated with lipid rafts can shuttle between the plasma membrane and internal depots in an anchorage dependent fashion; in this context Arf 6 is involved in the return of lipid rafts to the plasma membrane [34]. Other recent studies have found a link between the Rab family of small GTPases that regulate many aspects of membrane trafficking and the process of Rac activation. Thus Rab5 has been implicated in a key functional recycling of Rac that involves co-recruitment of Rac and the Rac-specific GEF Tiam1 to endosomal surfaces, followed by shuttling of Rac to the plasma membrane where it triggers actin meshwork formation [35]. These recent examples probably herald the emergence a rich new vein of information.

III RhoGAPs and their Role in Control of the Cytoskeleton and cell migration

Basic aspects of RhoGAPs

As discussed earlier, Rho GTPase GDP/GTP cycling is controlled by two classes of regulatory proteins that accelerate the otherwise very slow intrinsic guanine nucleotide exchange and GTP hydrolysis activity [36]. There are two major RhoGEF families, one comprised of the Dbl family (69 members) and the other the DOCK family (11 members) [37]. Similarly, the number of RhoGAPs (>70) also greatly outnumber the number of Rho GTPases [38,39]. That there exist multiple GEFs and GAPs, as well as effectors, for a single Rho GTPase reflects the critical role of these proteins as nodes in complex signaling networks.

Much recent evidence has uncovered a striking role for the mutation or aberrant expression of many RhoGEFs and RhoGAPs in a multitude of cellular processes and disease states, especially cancer and the related processes of cytoskeletal organization and cell migration [40]. For this review, we will focus on RhoGAPs and particularly the DLC family of RhoGAPs. All RhoGAPs share a conserved RhoGAP catalytic domain [38,39]. Otherwise, they show little sequence similarity in their remaining sequences, which typically contain multiple additional protein-protein and protein-lipid interacting domains, as well as many putative phosphorylation sites. These domains dictate the specific subcellular localization and regulation of each RhoGAP [39]. Such mechanisms of control allow high spatial and temporal control of the termination of GTPase activation, preventing inappropriate or prolonged signals. The spatial regulation of Rho activation in turn influences effector utilization, thus providing a critical mechanism of regulation of Rho GTPase function. Thus, the GAPs serve a critical signaling nodes, incorporating diverse cellular stimuli in order determine specific Rho GTPase outcomes.

In contrast to RhoGEFs, much less is known regarding RhoGAP regulation. A few RhoGAPs have been studied in molecular detail, including p190RhoGAP and Deleted in liver cancer-1 (DLC-1). For example, p190RhoGAP is tyrosine phosphorylated and binds to the RasGAP, p120RasGAP, both regulating its function [41]. However, very little is known about the full cellular regulation mechanisms of most of the RhoGAPs. This will be a great but important challenge in the Rho GTPase field, as different RhoGAPs likely play different and specific roles in different aspects of Rho GTPase function. Uncovering the signaling mechanisms that regulate RhoGAPs in specific cellular contexts will be necessary for a full understanding of Rho GTPase function. The future challenges will especially include an understanding of 1) which Rho GTPases are the targets of each RhoGAP *in vivo*, 2) which downstream signaling targets of each Rho GTPase do the GAPs affect, 3) which RhoGAPs are redundant and which are specific to certain Rho functions and 4) which Rho GTPases are most involved in disease processes and could serve as drug targets to specifically affect certain aspects of GTPase function without altering others. Below we discuss what is known regarding DLC-1 and related RhoGAPs.

DLC-1 in Cell Migration and Invasion

First identified as a gene deleted in liver cancer, subsequent studies found loss of DLC-1 gene expression in a wide variety of human cancers, including lung and breast [8]. Interestingly, the rate of heterozygous loss of DLC-1 in some cancer types approaches that of p53, underscoring its potential role as a tumor suppressor [42]. Additionally, recent genome-wide sequencing analyses of human tumors have identified missense mutations in DLC-1 [43,44]. Likewise, several studies have demonstrated that reintroduction of DLC-1 into liver, lung or breast cancer cell lines results in decrease tumorigenic growth [45-47], and in a recent mouse model of liver cancer, loss of DLC-1 together with Myc oncogene activation resulted in increased tumor growth [42]. Together, these results demonstrate an important role for DLC-1 in cancer and support a tumor suppressor role for DLC-1. DLC-2 and DLC-3 are highly related isoforms of

DLC-1 and similar less extensive observations also support their role as tumor suppressors [8]. Whether loss of DLC-2 and DLC-3 as well as that of DLC-1 must occur in cancer will be a subject of future investigation.

All RhoGAPs contain an ~150 amino acid RhoGAP catalytic domain [38,39]. DLC-1 and related isoforms contain two additional domains, an N-terminal SAM domain and a C-terminal START domain (Fig. 3). *In vitro*, we determined that the isolated RhoGAP domain acts as a potent GAP for RhoA, RhoB, RhoC, and to a lesser degree for Cdc42, but not Rac1 [48]. SAM domains (~70 amino acids) are putative protein interaction modules found in a diverse spectrum of signaling and nuclear proteins, typically as components of multi-domain proteins (e.g., Eph-related tyrosine kinases, Ets transcription factors). SAM domains have been shown to homo- and hetero-oligomerize, and can additionally bind both non-SAM domain-containing proteins. Structural studies of the SAM domain of DLC-2 suggest that it may bind lipids (although the specificity and *in vivo* relevance is unknown [49]). START domains (~120 amino acids) are lipid-binding domains found in proteins that transfer lipids between organelles. START domains are found in StAR, HD-ZIP and other signaling proteins. Representatives of the START domain family have been shown to bind different ligands such as sterols (StAR protein) and phosphatidylcholine.

Much less is known about the function of the SAM and START domains in DLC function. Our studies have found that the SAM domain appears to be involved in autoinhibition of the RhoGAP activity [50]. Between the SAM and RhoGAP domains is a long unstructured region that includes a phosphorylation-independent binding site (Y442 in human DLC-1) for the Src homology 2 domains of tensin family of adaptor proteins [51,52]. This binding region is necessary for focal adhesion localization as well as tumor suppression, suggesting that RhoGAP activity at or near focal adhesions and not total cell RhoGAP activity is necessary for tumor suppression. A phosphorylation site for rat DLC-1 has also been identified in this N-terminal region (S329), by activated AKT and RSK1 kinases, although the functional consequence of this modification has not been determined (Fig 3). Future work will involve understanding how DLC-1 is regulated by its additional domains and phosphorylation, as well as the precise the downstream mechanisms through which DLC-1 acts as a tumor suppressor.

Since aberrant activation of RhoA and RhoC has been implicated in oncogenesis, a logical hypothesis is that loss of DLC-1 function will lead to hyperactivation of these Rho GTPases, resulting in their stimulation of cell proliferation. Consistent with this possibility, two studies suggest that tumor suppression by DLC-1 works through RhoA [53,54], although our study demonstrated partial RhoGAP independent tumor suppression by DLC-1 [48]. Our studies also found that ectopic expression of DLC-1 in DLC-1 deficient lung tumor cells reduced the level of activated RhoA. Additionally, although FA association does not appear to alter DLC-1 RhoGAP activity *in vivo* or in vitro (unpublished results), FA association appears to be required for proper spatial regulation of Rho GTPases for tumor suppression.

As indicated above the Rho and Cdc42 GTPases play multiple critical roles in the regulation of actin organization, cell polarity, and cell migration. Thus the aberrant function of DLC-1 seen in many types of cancers would be expected to influence the activation state of these critical GTPases and to have significant effects on cytoskeletal organization and cell motility. We have recently explored this aspect in some detail [50]. Thus, overexpression of various activated forms of DLC-1 leads to profound changes in cytoskeletal organization with disruption of focal adhesions and the rapid, continuing and apparently random extension of long protrusions, as well as inability to retract the tail of the cell. These effects are likely mediated via reduction in Rho activity. Using single cell tracking assays, we have also found that overexpression of a form of DLC-1 (DLC-1ΔSAM) that lacks the SAM domain (and is thus activated), but which retains the remainder of the N-terminal region, has a profound effect

on cell motility. DLC-1ΔSAM increases the velocity of migration but reduces directionality; the overall result in bulk migration assays is a reduction in migration, presumably due to loss of directionality, an effect that may be mediated via DLC-1's inhibition of CDC42 and consequent effects on polarity. These effects on migration are not seen with active forms of DLC-1 that lack the N-terminal region. DLC-1ΔSAM is capable of associating with focal adhesions, but when it is expressed extensive disruption of these structures occurs. Thus it is not clear whether the profound effects of DLC-1ΔSAM on migration are due to its ability to localize to residual focal adhesions, or due to other interactions mediated through the N-termus.

Further evidence for a key role for focal adhesion localized DLC-1 comes from our studies of a truncated version of the protein (DLC-1N) that contains the N-terminal domain but not the GAP domain; we also constructed a second version with a mutation at the Y442 site (DLC-1N Y442A). DLC-1N retains the ability to localize to focal adhesions, but the version with the mutation at 442 does not. Overexpression of DLC-1N leads to almost complete paralysis of cell movement, whereas the 442 mutant had no effect. Using fluorescence microscopy and immunochemistry we have demonstrated that DLC-1N displaces endogenous DLC-1 from focal adhesions. This strongly argues that, not only GAP activity, but also proper focal adhesion localization of DLC-1, play important roles in the control cell migration. Presumably focal adhesion associated DLC-1 can orchestrate localized effects on Rho and CDC42 that are critical for control of cell velocity and directionality.

In addition to the work described above, a number of other studies have demonstrated that DLC-1 inhibits cell motility or invasiveness in cultures of liver, breast, ovarian and lung cancer cell lines [48,55–58]. Further, the observed effects of *DLC-1* on cell motility are consistent with its proposed role as a metastasis suppressor gene [8]. For example, using gene chip array analysis several groups have found that DLC-1 is unde-rexpressed in highly metastatic cells [55,59].

In summary, the RhoGAP DLC-1 and related family members play important roles both in the regulation of tumorigenesis and in associated cytoskeletal activities that impact on the motile, invasive and metastatic characteristics of cancer cells. Whether these two aspects of DLC-1 function occur through the same or different GTPase pathways will be an important goal of future research. The functions of DLC-1 seem to depend not only on its enzymatic activity as a GAP but also on its focal contact localization and its interactions with other proteins that regulate and localize its function.

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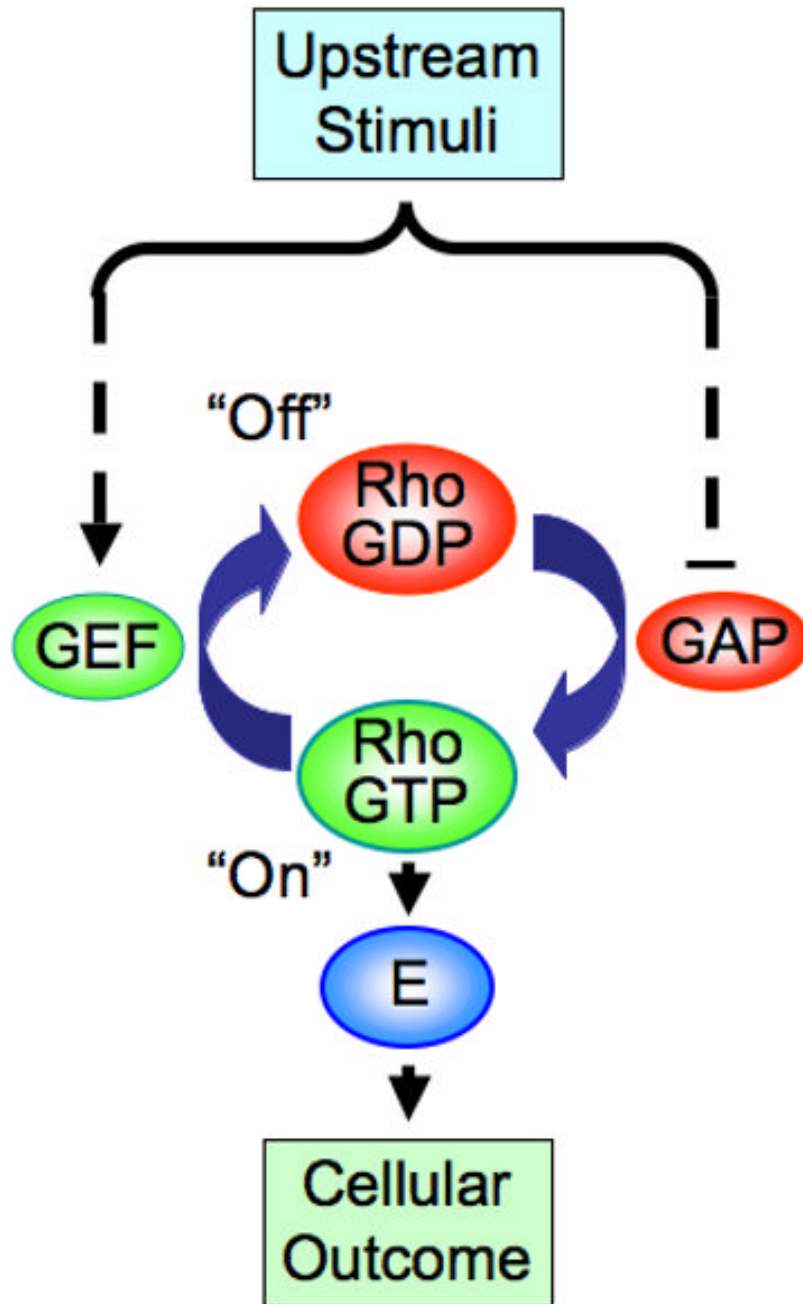
Figure 1. Rho GTPases function as GDP/GTP regulated on-off switches in signal transduction
Rho GTPases function as GDP/GTP regulated on-off switches in signal transduction. Rho GTPase cycle between an active GTP-bound and an inactive GDP-bound state. This cycle is regulated by RhoGEFs that promote GDP/GTP exchange and by RhoGAPs that stimulate hydrolysis of the bound GTP. Activated Rho GTPases preferentially associate with downstream effectors, which in turn regulate cytoplasmic signaling networks that control actin organization and other cellular processes. Extracellular stimulus activation of cell surface receptors cause activation of Rho GTPase primarily by activation of RhoGEFs. There is emerging evidence that stimuli can regulate Rho GTPase by controlling the activity of RhoGAPs.
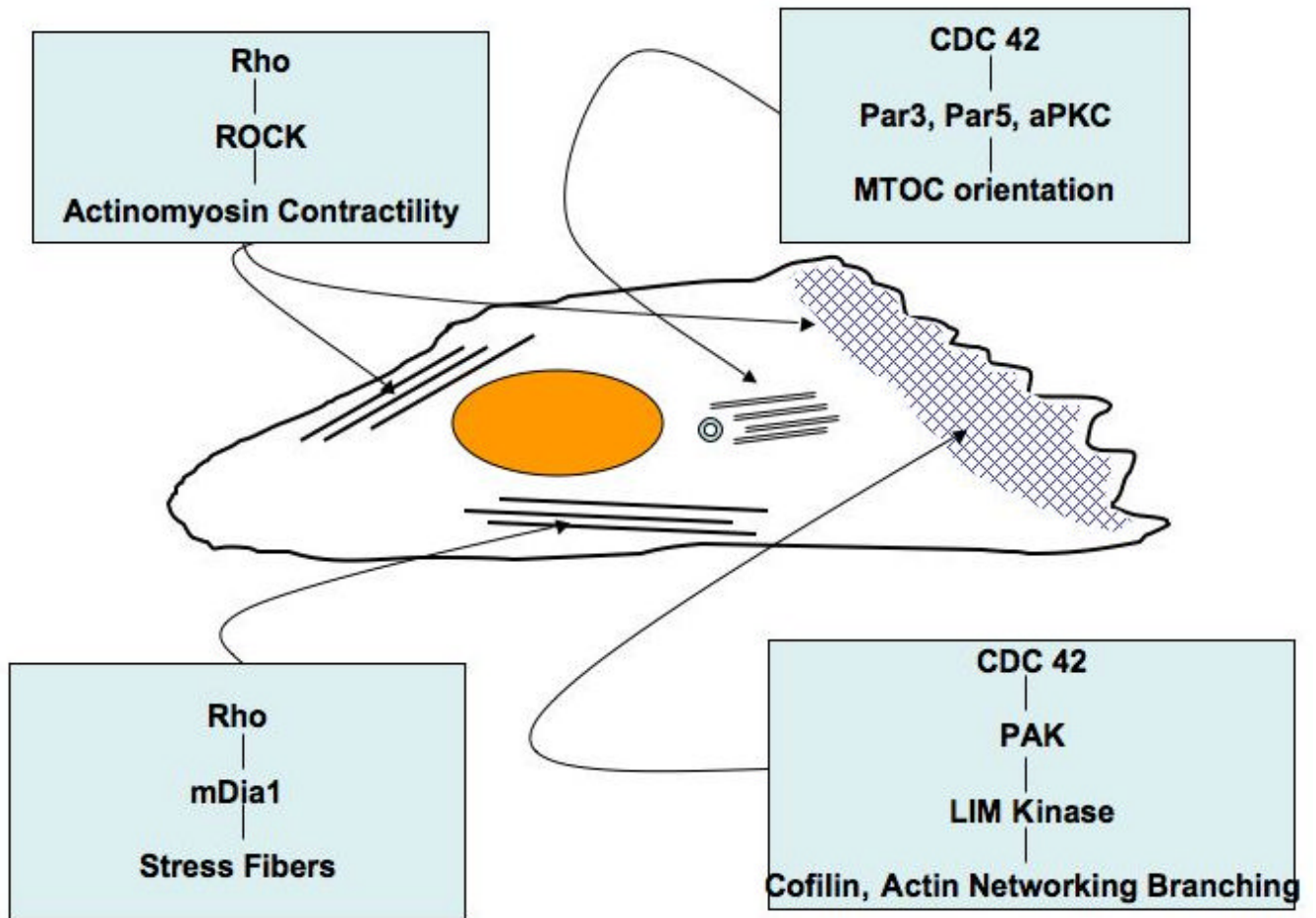
Figure 2. DLC-1 Affects the Cytoskeleton and Cell Motility Via Rho and CDC42
This figure illustrates key signaling pathways involving the Rho and CDC 42 GTPases that are implicated in control of the cytoskeleton, cell polarity and cell migration. Since DLC family proteins are GAPs for Rho and CDC42, they may influence all of these pathways.
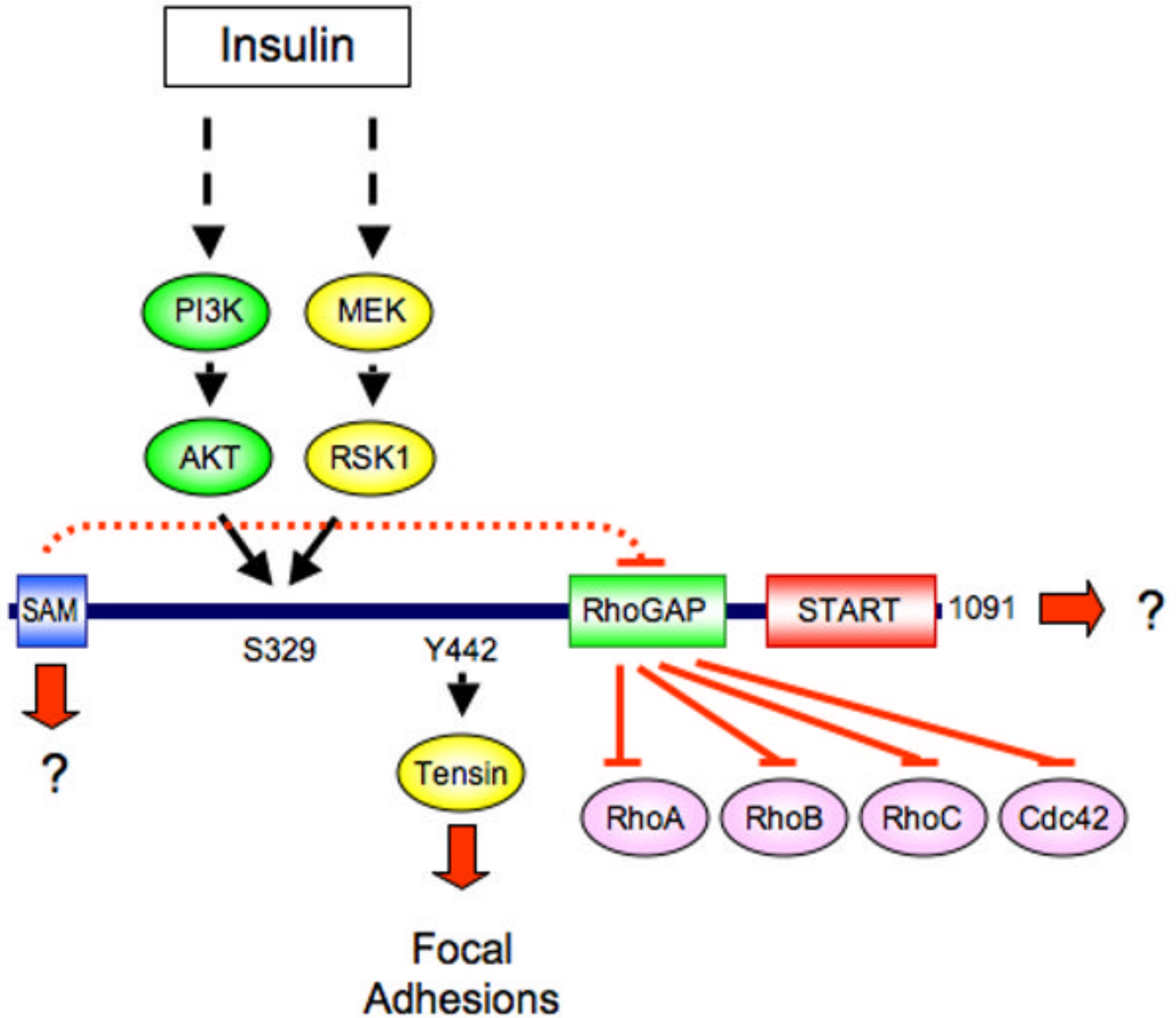
Figure 3. Interactions of DLC-1
Known interactions and functions of DLC-1. To date, biochemical and cellular analyses of both rat (p122RhoGAP) and human DLC-1 demonstrate that DLC-1 is a potent GAP primarily for RhoA and related isoforms. Additionally, there is also evidence for Cdc42 GAP activity. Our structure-function studies found that the SAM domain may serve as an autoinhibotory domain for the RhoGAP catalytic activity. Insulin-stimulated activation of the PI3K-AKT or MEK-ERK-RSK1 protein kinase cascades has been shown to cause phosphorylation at S322 in rat DLC-1 (S329 in human DLC-1). Two recent studies determined that Y422 in human DLC-1 was critical for this association; this residue is a component of a phosphorylation-independent motif that binds the Src homology 2 (SH2) domain of tensin family proteins (tensin-1, cten, etc.) that are associated with focal adhesions.