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Positive feedback between Dia1, LARG, and RhoA regulates cell morphology and invasion

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The Rho-effector Dia1 controls actin-dependent processes such as cytokinesis, SRF transcriptional activity, and cell motility. Dia1 polymerizes actin through its formin homology (FH) 2 domain. Here we show that Dia1 acts upstream of RhoA independently of its effects on actin assembly. Dia1 binds to the leukemia-associated Rho-GEF (LARG) through RhoA-dependent release of Dia1 autoinhibition. The FH2 domain stimulates the guanine nucleotide exchange activity of LARG in vitro. Our results reveal that Dia1 is necessary for LPA-stimulated RhoA activity. Interestingly, ΔDia1N caused substantial inhibition of RhoA (Fig. 1A). Active DAD-deletion mutants (ΔDAD) of mouse Dia1, Dia2, and Dia3 also stimulated RhoA but not Cdc42 activity, indicating that this is a shared characteristic among DRFs of this family (Fig. 1C). Dia1 also activated Rac as previously suggested (Tsujii et al. 2002, data not shown). Further analysis revealed that the FH2 domain was sufficient to activate the heterotrimeric G-proteins G12 and G13, which can bind to RGS-containing Rho-GEFs such as leukemia-associated Rho-GEF (LARG), initially isolated from a patient with acute myeloid leukemia (AML) (Kourlas et al. 2000; Vazquez-Prado et al. 2004). Rho-dependent mechanisms have emerged as critical processes in tumor progression (Sahai and Marshall 2002; Lozano et al. 2003), and evidence exists that Rho/ROCK function is essential to specify a unique type of actin-rich lamellipodia in tumor cell invasion (Sahai and Marshall 2002; Wyckoff et al. 2006). However, the role of DRFs in tumor cell behavior has not been investigated.

In this study, we provide evidence for an essential role of the Rho-effector Dia1 in LPA-mediated Rho/ROCK activity for tumor cell morphology and invasion that involves LARG, thereby constituting a positive feedback loop toward RhoA.

Results and Discussion

To explore the role of Dia1 in the regulation of RhoA, we performed GTPase activity assays (Goulimari et al. 2005). HEK293 cells were used to achieve transfection efficiencies >70% to determine whether versions of Dia1 lacking the regulatory N terminus (ΔDia1N) (Fig. 1A) required for autoinhibition could induce endogenous RhoA activity. Interestingly, ΔDia1N caused substantial activation of RhoA (Fig. 1B). Active DAD-deletion mutants (ΔDAD) of mouse Dia1, Dia2, and Dia3 also stimulated RhoA but not Cdc42 activity, indicating that this is a shared characteristic among DRFs of this family (Fig. 1C). Dia1 also activated Rac as previously suggested (Tsujii et al. 2002, data not shown). Further analysis revealed that the FH2 domain was sufficient to activate RhoA (Fig. 1D). Since the FH2 is also sufficient for actin assembly and activation of SRF, we tested their possible involvement, indicative of an indirect mechanism. For this we treated cells with Latrunculin B, which sequesters actin monomers and prevents SRF activation (Sotiropoulos et al. 1999). Although Latrunculin B effectively blocked FH2-induced SRF activation as expected (Fig. 1E), it failed to inhibit RhoA activation by FH2 and LARG, while transfection of C3 transferase as a control blocked RhoA activity (Fig. 1D). Interestingly and in agreement with previous findings (Copeland and Treisman 2002), we observed that FH2-induced SRF activation was significantly inhibited by C3 (Fig. 1E), suggesting that upstream RhoA contributes to FH2-exerted actin dynamics. The FH2 domain dimerizes involving its N-terminal lasso loop linker in order to nucleate actin filaments, whereas the core FH2 (FH2core) lacking the lasso behaves as a barbed end capper (Shimada et al. 2004; Otomo et al. 2005a). The FH2 domain of Dia1 promotes stress fiber formation and transcriptional activation of the MAL/SRF pathway through its actin-polymerizing activity (Copeland and Treisman 2002; Grosse et al. 2003; Miralles et al. 2003). A Dia1 mutant defective in FH2 dimerization interferes with lysophosphatidic acid (LPA)-induced stress fiber formation and SRF activity (Copeland and Treisman 2002), suggesting that Dia1 is part of LPA signal transduction known to play an important role in cell proliferation and metastasis of a variety of human cancers [Mills and Moolenaar 2003]. LPA receptors belong to the group of G-protein-coupled receptors that activate the heterotrimeric G-proteins G12 and G13, which can bind to RGS-containing Rho-GEFs such as leukemia-associated Rho-GEF (LARG), initially isolated from a patient with acute myeloid leukemia (AML) (Kourlas et al. 2000; Vazquez-Prado et al. 2004). Rho-dependent mechanisms have emerged as critical processes in tumor progression (Sahai and Marshall 2002; Lozano et al. 2003), and evidence exists that Rho/ROCK function is essential to specify a unique type of actin-rich lamellipodia in tumor cell invasion (Sahai and Marshall 2002; Wyckoff et al. 2006). However, the role of DRFs in tumor cell behavior has not been investigated.

In this study, we provide evidence for an essential role of the Rho-effector Dia1 in LPA-mediated Rho/ROCK activity for tumor cell morphology and invasion that involves LARG, thereby constituting a positive feedback loop toward RhoA.

Keywords: Diaphanous-related formins; RhoA; LARG; actin polymerization; LPA; Rho-kinase

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Diaphanous-related formins (DRFs) are Rho–GTPase-binding proteins that possess conserved functions in actin cytoskeleton dynamics exerted through their formin homology (FH) 2 domains (Goode and Eck 2007). DRFs are involved in essential cellular processes such as cytokinesis, cell movement, and polarity (Faix and Grosse 2006; Gomez et al. 2007), which are frequently deregulated during pathological situations like tumor cell transformation and metastasis [Sahai 2005]. The dormant conformation of the DRF Dia1 is maintained by intramolecular association of its regulatory N terminus to the diaphanous autoregulatory domain (DAD), which is relieved through binding of active RhoA [Lammers et al. 2005; Otomo et al. 2005a]. The catalytic FH2 domain is believed to become “exposed” by conformational changes in the DRFs to promote barbed end actin polymerization by forming a tethered dimer [Xu et al. 2004;
Therefore, we compared FH2core and FH2 for RhoA activity and actin polymerization. We found that both domains activate RhoA (Fig. 1F), but only the FH2 was able to stimulate SRF and actin polymerization, whereas the FH2core inhibited SRF and basal actin polymerization due to its capping activity (Fig. 1G,H). These data demonstrate that Dia1 stimulates RhoA activity independently of its actin nucleation activity, thus representing a novel function of this conserved protein.

Next we tested the possibility that Dia1 may be part of a signal transduction pathway toward RhoA. For this we stimulated cells with LPA under conditions in which Dia1 expression was suppressed by RNA interference. We observed that Dia1 is required for full RhoA activation by LPA as shown by siRNA. Cells were untreated or stimulated with LPA (10 µM, 5 min) as indicated, and activated RhoA was precipitated. Cell extracts were immunblotted for the indicated proteins. [I] HEK293 cells were transfected with Flag-Dia1ΔDAD and increasing amounts of expression plasmids encoding dnLARG [1.0, 5.0 µg]. Activated RhoA and cell extracts were immunblotted for the indicated proteins. All Western blot quantifications shown are the mean ± SEM of at least two independent experiments.

The facts that [1] LARG, like Dia1, is implicated in LPA signaling by interaction with Gβγ12/13 as well as by using a dominant-negative mutant of LARG [dnLARG] (Vogt et al. 2003; Tanabe et al. 2004; Vazquez-Prado et al. 2004], and [2] the Drosophila LARG homolog drhobeuf colocalizes with Diaphanous at the furrow canal during cellularization (Grosshans et al. 2005). To investigate whether LARG is involved in Dia1-induced stimulation of RhoA, we used the previously characterized dnLARG lacking the DH/PH domains [Fig. 2A; Vogt et al. 2003]. Despite relatively low expression levels, dnLARG partially but significantly inhibited Dia1–ΔDAD-induced and increasing amounts of expression plasmids encoding dnLARG [1.0, 5.0 µg]. Activated RhoA and cell extracts were immunblotted for the indicated proteins. All Western blot quantifications shown are the mean ± SEM of at least two independent experiments. 

Figure 1. Role of Dia1 in activation of RhoA. (A) Schematic representation of mDia1. (B) Cells were transfected with control plasmid or increasing amounts of plasmids (0.2, 0.5, 1.0 µg) encoding ΔDia1N and subjected to RhoA-GTP pull-down assays. RhoA and Flag-ΔDia1N were analyzed by Western blotting. (C) Activation of RhoA but not Cdc42 by ΔDAD expression plasmids for Dia–3 is shown. Active RhoA and Cdc42 were precipitated simultaneously from cell extracts. (D) FH2-induced RhoA activity in the absence or presence of 0.5 µM Latrunculin B (LatB) or in the absence or presence of C3 plasmid (0.5 µg). (E) Cells expressing FH2 (0.1 and 0.3 µg) together with the SRF reporter were cotransfected with C3 (0.1 µg) or pretreated with LatB as indicated. Reporter activity is shown as the mean ± SEM of three independent experiments. (F) FH2 or FH2core was transfected. The amount of Rho-GTP bound, of total RhoA, and of expressed FH2 and FH2core proteins was analyzed by Western blotting. (G) FH2 or FH2core capping and actin polymerizing activity using pyrene-actin assembly assays is shown. (H) Expression plasmids for FH2 or FH2core were transfected with an SRF reporter. Data represent the mean ± SEM of three independent experiments. (I) Dia1 is required for RhoA activation by LPA as shown by siRNA. Cells were untreated or stimulated with LPA (10 µM, 5 min) as indicated, and activated RhoA was precipitated. Cell extracts were immunblotted for the indicated proteins. (J) HEK293 cells were transfected with Flag-Dia1ΔDAD and increasing amounts of expression plasmids encoding dnLARG [1.0, 5.0 µg]. Activated RhoA and cell extracts were immunblotted for the indicated proteins. All Western blot quantifications shown are the mean ± SEM of at least two independent experiments.
Dia1 interacts with LARG. (A) Schematic representation of LARG. (RG) Regulator of G protein signaling, (DH) Dbl homology, (PH) pleckstrin homology. (B) Cells maintained in 10% serum were lysed before immunoprecipitation with α-Dia1 or control antiserum (co). Cell lysates and precipitates (IP) were analyzed by immunoblot using α-Dia1 antibody. (C) Lysates were prepared from cells expressing myc-LARG derivatives or full-length LARG. Immunoprecipitates (IP) prepared using α-Dia1 were analyzed for associated LARG derivatives (Co-IP) by immunoblot as indicated. (D) Lysates from cells expressing FH2core and LARG C-terminal mutants were immunoprecipitated and analyzed by immunoblotting as indicated.

Figure 2. Dia1 interacts with LARG. (A) Schematic representation of LARG. (RG) Regulator of G protein signaling, (DH) Dbl homology, (PH) pleckstrin homology. (B) Cells maintained in 10% serum were lysed before immunoprecipitation with α-Dia1 or control antiserum (co). Cell lysates and precipitates (IP) were analyzed by immunoblot using α-Dia1 antibody. (C) Lysates were prepared from cells expressing myc-LARG derivatives or full-length LARG. Immunoprecipitates (IP) prepared using α-Dia1 were analyzed for associated LARG derivatives (Co-IP) by immunoblot as indicated. (D) Lysates from cells expressing FH2core and LARG C-terminal mutants were immunoprecipitated and analyzed by immunoblotting as indicated.

Figure 3. Dia1 stimulates LARG GEF activity. (A) Actin assembly assays of Dia1-CT show release of NT-induced inhibition by RhoAV14. (B) His-Dia-NT and His-Dia-CT proteins were incubated with GST-LARG C-terminal regions, and complexes were analyzed by immunoblot using α-His antibody. Coomassie staining for GST-LARG CT-N and CT-C is shown in the bottom panel. (C) GST pull-downs show association of LARG-CT-C with Dia1-CT and the release of NT-induced binding inhibition by RhoAV14. Complexes were analyzed by immunoblot as indicated. Coomassie staining for GST-LARG CT-N and CT-C is shown in the bottom panel. One representative out of four independent experiments is shown. (D) Indicated concentrations of GST-LARG incubated with [3H]-GDP-loaded RhoA. [3H]-GDP-bound RhoA (%) is plotted against incubation time. Shown is the mean ± SEM of three independent experiments. (E) One nanomolar GST-LARG was incubated with [3H]-GDP-loaded RhoA with 10-fold molar excess of GST or with 10- and 100-fold molar excess of GST-FH2. [3H]-GDP-bound RhoA (%) is plotted against incubation time. Shown is the mean ± SEM of three independent experiments.
results show that Dia1 can stimulate the GEF activity of LARG. We propose that the Dia1/LARG interaction results in conformational changes of regulatory regions within the LARG C terminus.

LARG was identified as a gene fusion rearranged in AML (Kourlas et al. 2000). Up-regulated activities of RhoA have been reported for a variety of human cancers (Sahai and Marshall 2002; Lozano et al. 2003). Here we show that Dia1 promotes RhoA activity via interaction with LARG and that Dia1 is required for LPA-mediated RhoA activity. Since LPA is known to play an important role in tumor development and progression promoting cancer cell metastasis and invasion (Mills and Moolenaar 2003), we tested the role of Dia1 in tumor cell invasion. One mode of tumor cell invasion in three-dimensional (3D) matrices is represented by the rounded mode of motility, for which Rho/ROCK signaling is necessary and sufficient (Sahai and Marshall 2003; Wyckoff et al. 2006). After optimizing the conditions for small interfering RNA (siRNA) toward Dia1 (Fig. 4A,B), we used highly invasive MDA-MB-435 human cancer cells (Sellappan et al. 2004; Rae et al. 2006), which express Dia1, Dia2 [Fig. 4B], and Dia3 [data not shown], and tested their ability to migrate into LPA-containing 3D matrices when Dia1 or Dia2 expression was down-regulated. Interestingly, knockdown of Dia1 efficiently and significantly inhibited the total number of tumor cells that have invaded as well as the overall invasion distance (Fig. 4D,E, Supplementary Videos 1, 2), whereas random MDA-MB-435 cell migration under two-dimensional (2D) tissue culture conditions was slightly increased [Fig. 4C]. Knockdown of Dia2 did not inhibit cell invasion [data not shown]. Similar results were obtained in A375m2 melanoma cells [data not shown], which also use the rounded mode of motility (Sahai and Marshall 2003). We next determined the involvement of LARG in MDA-MB-435 cell invasion. For this, we transfected GFP-dnLARG, GFP alone, or cotransfected with LARG–1–820 as a control (transfection efficiencies were ~1%) and assessed the GFP-positive cells invaded into the Matrigel. This revealed that dnLARG inhibited cell invasion [Fig. 4F] but not random 2D migration [Fig. 4C]. Inhibition of ROCK with 10 µM Y27632 blocked invasion of MDA-MB-435 cells as expected, while treatment with the Src-kinase inhibitor PP1 had no effect [Fig. 4G]. Consistently, usage of siRNAs against LARG significantly reduced cell invasion [Fig. 4H,I]. Together, these data suggest that LARG is involved in cancer cell invasion.

In order to understand better the mechanisms underlying the dependence on Dia1 for tumor cell invasion, we analyzed the MDA-MB-435 cell morphologies in Matrigel. We observed that these cells use the rounded bleb-associated mode of motility [Supplementary Video 3], while in 2D culture these cells displayed a mesenchymal-like cell morphology [data not shown]. Analysis of F-actin using phalloidin staining revealed that untreated or control siRNA-treated MDA-MB-435 cells showed extensive formation of membrane blebs when contacting and invading Matrigel [Fig. 5B, Supplementary Video 4]. However, cells that were silenced for Dia1 [Fig. 5A] displayed strikingly different morphologies and lost their ability to promote membrane blebbing [Fig. 5B, Supplementary Video 5].

Bleb-associated cell invasion requires ROCK (Sahai and Marshall 2003) to control contractile force through myosin activity (Riento and Ridley 2003). When basal as well as LPA-stimulated phosphorylation of myosin light chain (MLC), a ROCK substrate (Riento and Ridley 2003), was determined in MDA-MB-435 cells in the presence or absence of siRNA against LARG or Dia1, we found that MLC phosphorylation was strongly reduced [Fig. 5C–E] in contrast to Dia2 siRNA [Fig. 5F]. To investigate whether Dia1 promotes ROCK activity through RhoA, we used HEK293 cells to efficiently express the F-H2 domain with ROCK-I before determining in vitro kinase activities using either MBP or MLC as substrates. Interestingly, we found that active Dia1 increased ROCK activity in a Rho-dependent manner [Fig. 5G]. These results clearly show that activation of ROCK by Dia1 leads to an increase in ROCK activity to promote the MLC phosphorylation required for bleb-associated cell invasion.

Dia1 is a well-established downstream effector of ac-
Our results presented here reveal an unexpected and essential role for Dia1 in the activation of the Rho/ROCK signal transduction pathway and subsequent bleb-associated cancer cell motility. We provide evidence that Dia1 is required and sufficient for full LPA-induced activation of RhoA and downstream ROCK signaling. This effect can be mediated through interaction of Dia1 with LARG. The data shown here imply a novel signaling module by which Dia1, in addition to its role as a downstream RhoA effector, can function upstream of RhoA. This constitutes a positive feedback mechanism (Fig. 5H) amplifying signal-regulated cellular effects such as tumor cell invasion by activating RhoA and its effector ROCK.

Materials and methods

Plasmids, siRNA, transfections, and cell-based assays
Plasmids were generated using standard procedures. All siRNA oligos were from IBA. GTPase pull-down assays, immunoprecipitations, and Rho-kinase and SRF activity assays were performed in HEK293 or NIH3T3 cells. See the Supplemental Material for details.

Protein purification, actin assembly, and guanyl-nucleotide exchange assays
Proteins were produced and purified in Escherichia coli strain DE3 as GST or His fusions. Actin polymerization was monitored as described (Brandt et al. 2007). Guanyl-nucleotide exchange assays were performed using GST-RhoA loaded with [8-3H]GDP. See the Supplemental Material for full details.

3D matrigel invasion and confocal analysis
Human MDA-MB-435 cancer cells were used for invasion assays. Assays were analyzed using confocal microscopy (Leica TCS SP2). See the Supplemental Material for full details.

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References


