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The RLIP76 N-terminus binds ARNO to regulate PI 3-kinase, Arf6 and Rac signaling, cell spreading and migration

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ABSTRACT

RLIP76 is a multifunctional protein involved in tumor growth and angiogenesis, and a promising therapeutic target in many cancers. RLIP76 harbors docking sites for many proteins, and we have found that it interacts with ARNO, a guanine nucleotide exchange factor for Arf6, and that RLIP76 regulates activation of Rac1 via Arf6, and regulates cell spreading and migration in an ARNO and Arf6-dependent manner. Here we show that ARNO interacts with the RLIP76 N-terminal domain, and this domain was required for RLIP76-dependent cell spreading and migration. We identified two sites in the RLIP76 N-terminus with differential effects on ARNO binding and downstream signaling: Ser29/Ser30 and Ser62. Ser29/30 mutation to Alanine inhibited ARNO interaction and was sufficient to block RLIP76-dependent cell spreading and migration, as well as RLIP76-dependent Arf6 activation. In contrast, RLIP76(S62A) interacted with ARNO and supported Arf6 activation. However, both sets of mutations blocked Rac1 activation. RLIP76-mediated Rac and Arf6 activation required PI3K activity. S29/30A mutations inhibited RLIP76-dependent PI3K activation, but S62A mutation did not. Together these results show that ARNO interaction with the RLIP76 N-terminus regulates cell spreading and motility via PI3K and Arf6, independent of RLIP76 control of Rac.

1. Introduction

Cell migration is a key component of development, wound healing and immunological responses [1,2]. It also plays essential roles in cardiovascular disease [3], tissue remodeling [4], angiogenesis [5] and invasion of primary tumor cells and subsequent metastasis to distant sites [6–8]. Migrating cells extend protrusions in the forward direction – driven by activation of the Rho family small GTPase Rac1 – and form new integrin-mediated attachments to the extracellular matrix (ECM) and release attachments at the rear to allow the cell mass to pull forward [1,9–11]. Rac1 is directly implicated in migration-dependent phenotypes including those listed above [12–18]. We identified the R-Ras effector protein, RalBP1/RLIP76 as an essential mediator of Rac1 activation, cell spreading and cell migration [19]. We have further demonstrated that RLIP76 mediates Rac1 activation through regulation of Arf6, and that RLIP76 physically interacts in cells with an activator of Arf6, ARNO [19]. Rac1 and Arf6 are GTP-binding proteins, and Guanine nucleotide binding modulates the enzyme activation state [20–24]. Thus, the cellular functions of Rac1 and Arf6 are tightly controlled spatially and temporally by regulation of their nucleotide-bound state, which in turns controls their effects on cell spreading and migration.

RLIP76 (Ral-interacting protein of 76 kDa, also Ral-binding protein 1 or RalBP1) is an effector of activated Ral GTPase, and regulates endocytic recycling, actin cytoskeleton remodeling in cells and during gastrulation, and is a transporter of glutathione conjugates and chemotherapeutic agents such as doxorubicin; this proposed function has been linked to resistance to apoptosis (via outward transport of xenobiotics) and to cancer progression [25–32]. The RLIP76 polypeptide contains an N-terminal domain of undefined function, a central RhoGAP domain which inhibits Rac1 and Cdc42 *in vitro*, a Ral-binding domain (RBD), and a C-terminal region, also of undefined function, predicted to adopt a coiled-coil structure [29,33]. We identified RLIP76 as a novel effector of the pro-migratory GTPase, R-Ras [19,34], and we further

Abbreviations: GAP, GTPase activating protein; GGA3, golgi-localized, γ-ear-containing Arf-binding protein 3; HA, hemagglutinin; PBD, p21-binding domain; PI3K, phosphatidylinositol (3)-kinase; PIP_3, phosphatidylinositol (3,4,5) trisphosphate.

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showed that RLIP76 is downstream of R-Ras in promoting cell spreading and cell migration [19]. We have found that the role of RLIP76 in spreading and migration is to regulate activation of Rac1 and a related upstream GTPase, Arf6, a class III ADP-ribosylation factor involved in cell migration. [19]. Rac1 and Arf6 can be activated through multiple mechanisms, including adhesion-induced integrin signaling. We have shown that depletion of RLIP76 blocks attachment-induced rapid activation of both Rac1 and Arf6 in cells, and enforced activation of Rac1 by expression of GTP-locked Rac1(G12V) overcame the spreading defect in RLIP76-depleted cells [19]. Thus, RLIP76 regulates Rac1 and Arf6 to promote spreading and migration. In this study we describe a molecular mechanism through which ARNO binds the RLIP76 N-terminus to affect Arf6-dependent but Rac1-independent cell spreading and migration.

2. Materials and methods

2.1. Cell culture, transfections, immunoprecipitations and Western blotting

NIH 3T3 cells were cultured and transfected, and processed for immunoprecipitations and Western blotting as described [35].

2.2. Antibodies, cDNAs and reagents

Antibodies purchased were: GFP (B-2), His-probe (H-15), GST (B-14) and Arf6 (3A-1), Santa Cruz Biotechnology; HA (16B12), Covance; Rac (23A8), Millipore; Akt, phospho-Akt (phosphoSer473), Invitrogen; secondary antibodies, LICOR. FLAG- and GST-ARNO were gifts from Lorraine Santy (Penn State University, State College, PA). Human RLIP76 cDNA containing a 5′ HA tag was as described [19]. HA-tagged RLIP76(ΔN) truncation plasmid was generated by PCR from the HA-RLIP76(mismatch)/pcDNA3.1(−) template using the following primer set: 5′-GGAGATATCGGCGTCATGTACCCATACGATGTTCCAGATTACGCTCTCGAGCCAATTCAGGAGCCAGAGGTGC-3′ and 5′-CGCAACCTTGCTCAGATGGACGTCTCCTTCCTATCCCTGCTGGG-3′. A RLIP76 N-terminal fragment (corresponding to amino acids 1–192) was generated by subcloning an EcoRV/EcoR1 fragment from the full-length template into the pEGFP-C3 and pcDNA3.1(−) vectors using complementary sites. A C-terminal fragment (corresponding to amino acids 443–655) was generated by PCR from the full-length template using the following primer set (5′-GCCCTCAAAAGAGAATTCAGAGAAGCTAAAAGACAGG-3′ and 5′-GGGGGTACCTGCGGCCGCTCAGATGGCCGTCTCCTTCCTATCC-3′), and subcloned into the pEGFP-C3. A ΔNΔC fragment was generated from the full-length template using the ΔN forward strand primer and the ΔC reverse strand primer above. Point mutations were made on the full-length template background by Quikchange mutagenesis (Stratagene) according to the manufacturer's instructions, using the following primers with corresponding reverse complement primers: S29/30A, 5′-GGGCTTACCCGGACCCCCGCCGCTGAAGAGATCAGCCCTACTAAGTTTCCTGG-3′; S62A, 5′-CCTCCATGAGCCTCCTGATGTAGTGGCTGATGATGAGAAAGATCATGGGAAG-3′. An RLIP76 shRNA targeting plasmid was as described previously [36].

2.3. Cell spreading and migration

Cell spreading and migration were assessed as described previously [35,36].

2.4. Rac activation

Rac and Arf6 activation were monitored by pulldown with GST-PBD or GST-GGA3 proteins as described previously [19,37].

2.5. Statistical analysis

One-way ANOVA followed by Fisher PLSD analysis was used for all statistical data analysis, using StatView (SAS). A 5% probability was considered significant. All western blots and cellular assays are representative of at least three independent experiments.

3. Results

3.1. The Arf exchange factor ARNO interacts with the RLIP76 N-terminus

We previously found that RLIP76 interacts with ARNO, a guanine nucleotide exchange factor for Arf1 and Arf6 [19]. We first mapped the ARNO interaction domain in RLIP76, by co-immunoprecipitation (IP) of FLAG-tagged ARNO with truncated forms of HA-tagged RLIP76. ARNO was enriched in IPs of full-length HA-RLIP76 (1-655), consistent with previous observations (Fig. 1A) [19], as well as IPs of truncated HA-RLIP76 lacking the C-terminal 293 amino acids, which comprise the Ral-binding domain (RalBD) and C-terminal domain (1–392); thus, ARNO interacts with RLIP76 in a region that does not include the RalBD or C-terminus. However, ARNO did not co-precipitate with RLIP76 lacking the N-terminal 180 amino acids (RLIP76 (Δ180)). This region comprises the N-terminus of RLIP76 up to the N-terminal border of a putative helical domain as predicted by secondary structure modeling [38,39]. Furthermore, the first 192 amino acids of RLIP76 (1–192) were sufficient to interact with ARNO (Fig. 1A). Thus, the RLIP76 N-terminus is necessary and sufficient for ARNO interaction.

We considered how this interaction may be regulated, and noted a mass spectroscopy study from Herlevsen et al., which identified multiple Ser residues as utilized phosphorylation sites within the RLIP76 N-terminus, most prominently at Ser29, 30, 62, 92 and 93 [40]. We mutated each of these Ser residues (singly, or in pairs in cases of adjacent Serines) to Ala to prevent phosphorylation [41], and identified two sets of residues with distinct ARNO-binding profiles. RLIP76 harboring Ser (S) 29/30-Ala (A) double mutation (S29/30A) did not interact with ARNO. In contrast, ARNO co-precipitated with RLIP76 (S62A) (Fig. 1B). As before, deletion of the RLIP76 N-terminus (ΔN) blocked ARNO interaction, whereas C-terminal truncation (ΔC) did not. Thus, RLIP76 interaction with ARNO at the RLIP76 N-terminus requires Ser29 and 30 but not Ser62.

3.2. RLIP76 N-terminal domain Serine residues are required for cell spreading and migration

RLIP76 expression was suppressed by co-transfection with a plasmid encoding a RLIP76-targeting short hairpin RNA (shRNA) [19,36], and with either GFP as a transfection marker, or GFP-RLIP76 variants harboring silent mutations in the shRNA target sites to allow ectopic RLIP76 mutant expression against a background of silenced endogenous expression. Cell spreading was inhibited by RLIP76 depletion, and partially restored by reconstitution with full-length RLIP76, as we have shown previously [19] (Fig. 2A). In contrast, truncated RLIP76(ΔN), as well as RLIP76 truncated at both termini (ΔNΔC), each failed to restore spreading in the knockdown (GFP-positive) cells, indicating that the N-terminal domain of RLIP76 is required for cell spreading. However, RLIP76 lacking the C-terminal domain restored spreading in the knockdown cells, consistent with our previous findings that a C-terminally truncated fragment also lacking the Ral-binding domain (aa 1–392) is able to support cell spreading [19]. Interestingly, expression of full-length RLIP76 with the S29/30A mutation within the N-terminal domain did not restore spreading in RLIP76-depleted cells,

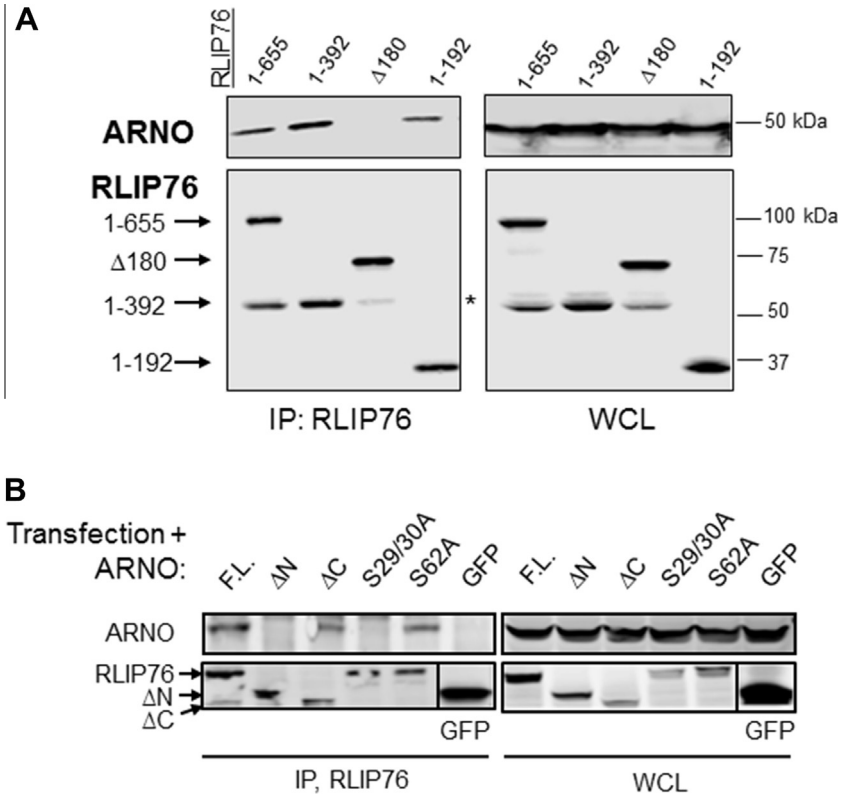

Fig. 1. ARNO binds the RLIP76 N-terminus via Ser29/30. (A) FLAG-ARNO was co-transfected with HA-tagged RLIP76 constructs in NIH 3T3 cells as indicated. HA antibody immunoprecipitates (IP) were probed with HA and FLAG antibodies to detect the presence of RLIP76 and truncated proteins and ARNO, respectively. *, α-HA-reactive polypeptide fragments or non-specific bands. WCL, whole cell lysate fractions. (B) GFP IPs and WCL fractions of cells transfected with GFP-RLIP76 variants or GFP control and FLAG-ARNO.

indicating that the S29/30 residues are important for RLIP76-dependent spreading in these cells (Fig. 2A). However, S62A mutation did not affect the ability of full-length RLIP76 to restore spreading in the knockdown cells (Fig. 2A). Thus, residues S29 and S30, but not S62A, are required for RLIP76-dependent cell spreading.

We next assessed migration of the RLIP76 knockdown/reconstituted cells using modified Boyden chambers. RLIP76 depletion substantially blocked migration through the filters, and migration was restored by reconstitution with full-length, WT RLIP76, as in previous studies [19]. However, RLIP76(S29/30A) substantially impaired migration (Fig. 2B and C), consistent with a blockade in cell spreading by this mutant. In contrast, RLIP76(S62A) – which supported cell spreading (Fig. 2A) – also restored migration to WT levels (Fig. 2B and C). Thus, Ser29 and 30 are required for RLIP76-dependent cell spreading and migration, whereas Ser62 is dispensable for these functions.

3.3. S29/30 and S62 are important for RLIP76 regulation of Rac and Arf6 activation

We next investigated the mechanisms by which the RLIP76 N-terminus regulates cell spreading and migration. We have previously demonstrated that RLIP76 regulates spreading through activation of Arf6 and Rac1 [19]. We evaluated adhesion-induced activation of Rac and Arf6 in RLIP76 knockdown/reconstituted cells. As expected, RLIP76 depletion blocked adhesion-induced activation of both Rac (Fig. 3A) and Arf6 (Fig. 3B), and both were restored in cells reconstituted with WT RLIP76. In contrast, RLIP76(S29/30A) and (S62A) were not able to restore Rac activation (Fig. 3A). Similarly, RLIP76(S29/30A), which was unable to support spreading and migration (Fig. 2), also did not restore Arf6 activation (Fig. 3B). However, RLIP76(S62A) restored Arf6 activation in knockdown cells (Fig. 3B), correlating with an ability of this RLIP76 mutant to restore spreading and migration (Fig. 2). Thus, Ser 29 and 30 are required for RLIP76-dependent Rac and Arf6 activation, but RLIP76 can support spreading and migration through an Arf6 pathway that does not require Rac activation.

3.4. RLIP76. regulates Rac and Arf6 activation via PI 3-kinase

The ability of RLIP76 to regulate cell spreading through Rac-dependent and -independent pathways led us to investigate a mechanism for RLIP76 regulation of Rac. We and others have previously found that RLIP76 expression is required for activation of phosphatidylinositol (3)-kinase (PI3K) in cells [42,43], an upstream regulator of Rac. RLIP76(S62A) was able to support PI3K activity, as measured by phosphorylation of the downstream target, Akt, whereas PI3K activity was suppressed in the presence of RLIP76(S29/30A) (Fig. 4A). To investigate a potential mechanistic link between RLIP76, PI3K, and Rac and Arf6, we determined the effects of PI3K blockade with wortmannin, on Rac and Arf6 activation by RLIP76. Whereas RLIP76 promoted adhesion-induced Rac activation, treatment with wortmannin abrogated RLIP76-dependent Rac activation (Fig. 4B). Thus, PI3K activation is downstream of RLIP76 and is required for RLIP76-dependent activation of Rac. As shown in Fig. 4C, wortmannin also suppressed Arf6 activation in RLIP76-reconstituted cells, indicating that RLIP76 regulates Arf6 through PI3K-dependent pathways. Together these results demonstrate that the N-terminal domain of RLIP76 is responsible for its roles in cell spreading and migration through an ARNO/Arf6 pathway downstream of PI3K but independent of its effects on Rac.

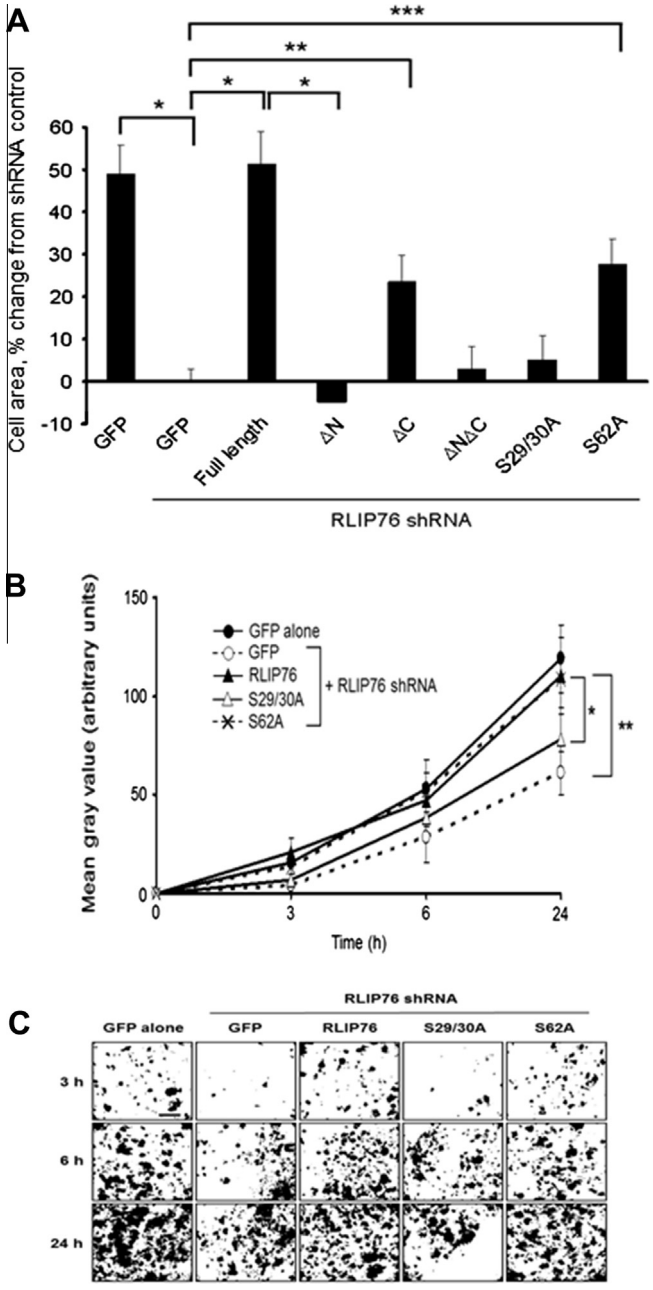

Fig. 2. Ser29 and 30 in the RLIP76 N-terminal domain are required for cell spreading and migration. (A) Cell spreading. Cells were co-transfected with GFP or GFP-RLIP76 variants and RLIP76 shRNA as indicated, and seeded on fibronectin-coated surfaces for 45 min, then fixed and imaged. The average surface areas of GFP-positive cells are shown +s.e.m. (A) $^{*}p < 0.0001$; $^{**}p < 0.003$; $^{***}p < 0.005$. (B) $^{*}p < 0.05$. $n = 3$. (B) Cell migration. Cells were co-transfected with GFP or GFP-RLIP76 variants and RLIP76 shRNA as indicated. At the indicated times after seeding cells on the tops of filters in modified Boyden chambers, the undersides of the filters were fixed and stained with crystal violet solution. Staining intensities, indicating densities of migrated cells in duplicate wells from three independent experiments are shown. (C) Representative images of the filters from (B). $^{*}p < 0.05$; $^{**}p < 0.03$. $n = 3$.

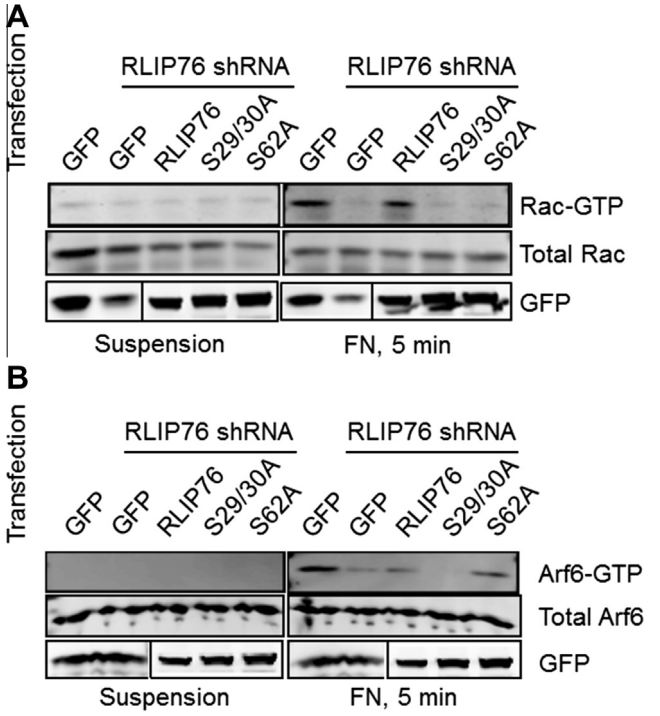

Fig. 3. RLIP76 S29/30 and S62 sites regulate the activation of Rac and Arf6. NIH3T3 cells transfected with GFP vector or GFP-RLIP76, S29/30A, or S62A, and RLIP76 shRNA as indicated were serum starved, and adhesion-induced activation of Rac (A) and Arf6 (B) was assessed as described in Section 2. Rac-GTP and Arf6-GTP were detected by immunoblotting and compared with total Rac and Arf6. FN, fibronectin.

4. Discussion

The results in this study outline a signaling pathway through which the multifunctional adapter protein RLIP76 regulates cell spreading and motility. We found that the RLIP76 N-terminus is the binding domain for the Arf-GEF ARNO, and Ser29/30 in RLIP76 was required for N-terminal/ARNO interaction. Ser29/30 were required for cell spreading and migration, ARNO interaction, and Arf6 and Rac activation. We further found that RLIP76 regulates PI3K activity, and Ser29/30 are required for this function of RLIP76 and downstream effects on Rac. However, Ser62 was also required for Rac activation, but not for PI3K or Arf6 activation, ARNO interaction, cell spreading, or cell motility. Thus, the distinct effects of these residues in the RLIP76 N-terminus point to a new mechanism for RLIP76 in cell spreading and motility: ARNO interaction with the RLIP76 N-terminus supports Arf6 activation, downstream of PI3K, which is necessary and sufficient for RLIP76-dependent cell spreading and migration, independent of RLIP76 regulation of Rac.

The structural basis of RLIP76 N-terminus/ARNO interaction is unknown. We found three Serine residues in the N-terminus – 29, 30, and 62 – which when mutated to Alanine modulated ARNO interaction in cells. These residues have been identified by mass spectrometry as utilized phosphorylation sites in cells [40]. Thus, one potential regulatory mechanism may involve phosphorylation at these sites. ARNO contains a coiled-coil domain, and the RLIP76 N-terminus may contain helical structures [44], which could support helix–helix-based interaction. Indeed, the RLIP76/Ral binding face does not resemble the typical Ras/Ral-association domain/effector structure but is primarily supported by helical packing [45]. The finding that S29/30A mutations in the N-terminus blocked ARNO interaction, whereas S62A mutation did not, suggests either a binding site localized around residues 29 and 30 but away from S62, or perhaps a more subtle level of regulation by selective phosphorylation, or another unknown mechanism involving these specific sites.

The selectivity of Ser29/30, but not Ser62, for ARNO interaction correlated with the effects of these mutations on RLIP76-dependent cell spreading and migration, and also correlated with effects on PI3K and Arf6 activation, but not Rac activation, pointing to divergent RLIP76 signaling pathways. However, both Rac and Arf6 required PI3K downstream of RLIP76. Together, these findings

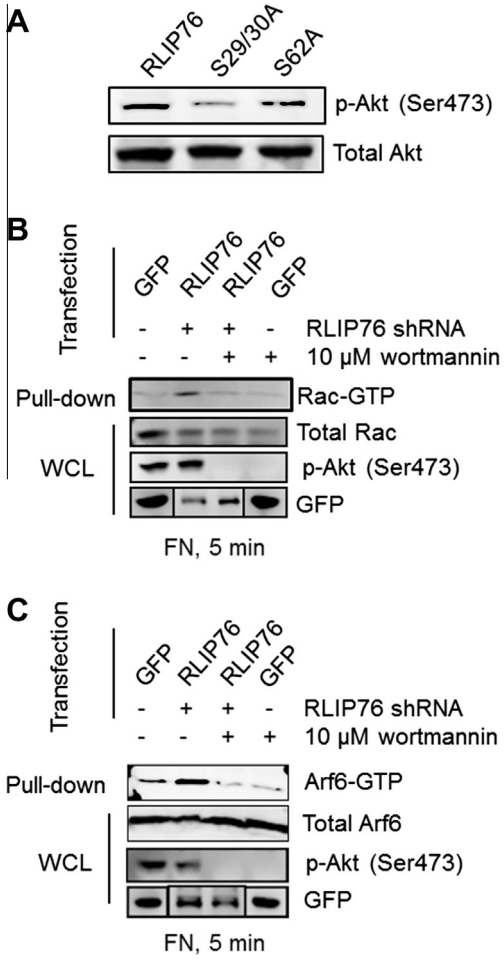

Fig. 4. RLIP76 regulates Rac and Arf6 activation via PI3K. (A) Cells were transfected with RLIP76 variants and RLIP76 shRNA as indicated, and PI3K activity was monitored by immunoblotting cell lysates with p-Akt (Ser473) and total Akt antibodies. (B) Rac activation and (C) Arf6 activation in cells transfected with RLIP76 and RLIP76 shRNA as indicated, in the presence of wortmannin (10 μM, +) or DMSO vehicle (−).

demonstrate that RLIP76 is capable of supporting cell spreading and migration in the absence of Rac activation, but only ARNO interaction and PI3K-dependent Arf6 activation are required. Hence, we propose that RLIP76 regulates spreading and migration through an ARNO/Arf6 pathway, independent of the role of RLIP76 in Rac activation. We previously found that constitutively activate Rac(G12V) could overcome the spreading defect in RLIP76-depleted cells [19], by stimulating Arf6. This may reflect the idea that generation of multiple lamellipodia, as in the case of Rac(G12V), enhances spreading but reduces directional migration; Arf6, in contrast, may assist in polarizing lamellipodial formation to stimulate directed migration [46,47]. Thus, whereas activated Rac can enhance spreading by activating Arf6 in the absence of RLIP76, Rac is not required for RLIP76-dependent spreading and migration so long as Arf6 activation is maintained, such as in the case of RLIP76(S62A).

How RLIP76 regulates Arf6 downstream of PI3K remains to be explored. One intriguing possibility relates to the role of RLIP76 as an effector of the small GTPase R-Ras [19]. We have found that R-Ras recruitment to the plasma membrane is required for its interaction with PI3K (also an R-Ras effector), localized generation of phosphatidylinositol (3,4,5) trisphosphate (PIP_3), and formation of membrane ruffles and cell spreading [35]. ARNO and other Arf GEFs are recruited to the plasma membrane by interaction with PIP_3 via their pleckstrin homology (PH) domains, leading to localized Arf6 activation which may in turn be the downstream driver of cell spreading and motility [48–52]. Arf6 activation at the plasma membrane may also drive a feed-forward loop by directly recruiting ARNO [53]. Thus, RLIP76 may participate in coupling R-Ras-dependent PIP_3 generation to recruitment of ARNO, leading to Arf6 activation and cell spreading. Elucidating these connections will be important to reveal the complete molecular mechanism of RLIP76 regulation of cell shape and motility. Taken together, our results demonstrate a new mechanism for RLIP76 in cell spreading and motility: ARNO interaction with the RLIP76 N-terminus, regulates Arf6 activation, downstream of PI3K, leading to RLIP76-dependent cell spreading and migration, independent of RLIP76 regulation of Rac.

Acknowledgments

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