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RIN1 regulates cell migration through RAB5 GTPases and ABL tyrosine kinases

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Stimulation of a receptor tyrosine kinase (RTK), such as EGFR, leads to RAS activation followed by RIN1 activation. RIN1, in turn, activates RAB5 family GTPases, as well as ABL tyrosine kinases. As expected, RIN1 expression directly correlates with RAB5-mediated EGFR endocytosis. We previously showed that normal receptor endocytosis and internalized EGFR fate also depend on the ability of RIN1 to concomitantly activate ABL tyrosine kinases, consistent with the established role of ABL kinases in cytoskeleton remodeling and the growing evidence that such remodeling plays a role in endocytic processes. Here we report that growth factor-directed cell migration, a physiological process that involves receptor endocytosis and actin remodeling, also requires the ability of RIN1 to coordinate RAB5 GTPase and ABL tyrosine kinase pathways.

RIN1 is a RAS effector protein.[1] Through a guanine nucleotide exchange factor (GEF) domain,[2] RIN1 activates RAB5 GTPases that promote early endosome maturation.[3] In addition, RIN1 directly binds to and activates ABL tyrosine kinases.[4,5] RIN1 associates directly with activated EGFR[6] and binds STAM proteins that escort receptors to lysosomes for degradation.[7] RIN1 sub-cellular localization, and hence its availability for interactions, is regulated by binding 14-3-3 proteins that favor cytoplasmic positioning.[8]

Overexpression of RIN1 in HeLa cells increased RAB5 activity and enhanced EGFR degradation compared with control cells, while silencing endogenous RIN1 had the opposite effect.[9] The RIN1 GEF domain mutant RIN1^{E574A}, which cannot activate RAB5 but can still activate ABL, suppressed RAB5 activity in a dominant negative manner and blocked EGFR degradation while it promoted receptor recycling.[9] This confirmed a positive role for RAB5 stimulation in receptor internalization and was consistent with a role for ABL in protecting activated receptors from ubiquitylation and internalization.[10,11]

We further demonstrated that RIN1 undergoes EGF-dependent phosphorylation on Tyr^{36}, emphasizing engagement of the RIN1→ABL pathway following receptor stimulation.[9] RIN1^{QM}, a mutant that cannot activate ABL but still activates RAB5, caused dominant suppression of EGF-induced ABL kinase activation and accelerated EGFR degradation.[9] HeLa cells expressing this mutant also showed greater RAB5 activation (**Fig. 1**) than did cells overexpressing wild type RIN1, implying that the RIN1→ABL pathway normally modulates the RIN1→RAB5 pathway during endocytosis.

The influence of RIN1 signaling on EGFR degradation suggested the involvement of CBL, an E3 ubiquitin ligase for EGFR, because receptor ubiquitylation drives sorting and degradation.[12] RIN1 overexpression increased CBL levels, consistent with RIN1 promoting EGFR::CBL interactions and accelerated EGFR degradation.[9] RIN1^{QM} dominantly blocked ABL kinase activity and enhanced CBL protein recruitment to EGFR,[9] supporting a negative role for RIN1→ABL signaling during CBL recruitment to EGFR.

We noted that RIN1^{QM} cells exhibited EGF-dependent macropinocytosis. In addition, EGF colocalized with macropinocytic membrane ruffles,[9] demonstrating that an imbalance in RIN1 effector pathways can shift the mode of RTK endocytosis. Blocking RIN1→RAB5 signaling stabilized EGFR and enhanced recycling, while blocking RIN1→ABL caused enhanced macropinocytosis with increased receptor degradation.[9] Notably, RAB5 activation has been linked to macropinocytosis through RAC1.[13,14]

Because receptor internalization and downregulation are directly relevant to chemotaxis, we examined the physiological significance of coordinated RAB5 and ABL signaling on cell migration toward a growth factor gradient. HeLa cells overexpressing wild type RIN1 showed a reduced rate of migration toward EGF, which was likely due to increased ABL activity.[15] Expression of RIN1^{E574A} (activates ABL but not RAB5) also decreased migration toward EGF, compared with cells expressing wild type RIN1 (**Fig. 2A**).

In contrast, HeLa cells expressing RIN1^{QM} showed significantly higher basal motility (no EGF gradient) compared with wild type RIN1 cells and, despite having less surface EGFR, migration toward EGF was similar to cells expressing wild type RIN1 (**Fig. 2B**). Hence the loss of RIN1→ABL signaling, while

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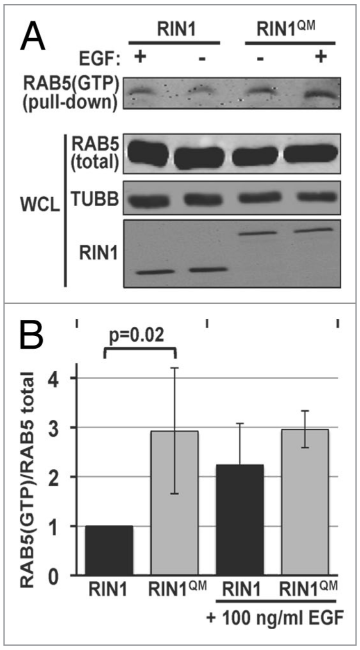

Figure 1. RIN1^{QM} shows enhanced RAB5 Activation. (**A**, top) Immunoblots of activated RAB5 in RIN1 and RIN1^{QM} HeLa cells untreated (–) or stimulated with 100 ng/ml EGF for 5 min (+). Alternate lanes were left blank to prevent merging of the lanes. Note that EGF addition samples are not alternating. (Bottom) Whole cell lysates of RIN1 and RIN1^{QM} HeLa cells from above, immunoblotted for total RAB5, Tubulin and RIN1. (**B**) Quantification of relative activated RAB5, normalized to total RAB5 from (**A**). The RIN1^{QM} protein includes multiple carboxy terminal tags that increase it molecular mass.

RIN1→RAB5 signaling persists, can enhance directed cell motility. This observation is consistent with macropinocytotic membrane ruffling and RAB5 activity being major contributors to cell migration, and suggests a mechanistic connection between RAS and RAC signaling pathways, as previously suggested.[13,14] The macropinocytic and migratory phenotypes we observed also emphasize a role for RIN1 in actin cytoskeleton remodeling, a function well-established for ABL tyrosine kinases[16] and strongly implied for RAB5 GTPases.[13]

The role of RIN1 signaling through RAB5 and ABL pathways in cell migration (this work) should be considered in the context of our previous work showing that BIN1, a BAR domain containing membrane-bending protein, is a RIN1 binding partner.[9] RIN1's proline-rich region mediates this interaction, possibly through the BIN1 SH3 domain as reported for in vitro binding of RIN3 to BIN1.[17] EGF stimulation enhanced the RIN1::BIN1 interaction, suggesting that RIN1 recruits BIN1 to facilitate receptor endocytosis during migration. In addition, RIN1 subcellular localization appeared to influence receptor internalization, as revealed using RIN1^{S351A}, a mutant with reduced 14-3-3 binding.[9] Localized receptor responses are fundamental to efficient directional cell migration. We have proposed a working model to explain the broad integration of signaling pathways through RIN1 following receptor tyrosine kinase activation.[9]

Our findings demonstrate a key role for RIN1 as a regulator of membrane trafficking and cytoskeleton remodeling during receptor endocytosis and cell migration. A deeper mechanistic understanding will require examination of other endocytic events in epithelial cells. For example, invasive bacteria, such as *Salmonella* and *Listeria*, enter epithelial cells through macropinocytosis and receptor-mediated phagocytosis, respectively.[18] RAB5 is a major player in both these entry mechanisms and functions in subsequent steps required for bacteria replication.[19,20] ABL kinases have also been implicated in the entry of invasive bacteria.[21] A role for RIN1 during pathogen entry could lead to identification of novel host cell drug targets for combating drug-resistant bacteria. Outside of epithelial cells, RIN1 is found most prominently in mature forebrain neurons where it inhibits certain types of learning.[22] A deeper understanding of RIN1 signal orchestration may shed light on synaptic plasticity mechanisms. How RIN1 coordinates GTPase and tyrosine kinase pathways also has direct implications for treatment of cancers with RTK and RAS oncogene involvement.

Materials and Methods

Cell culture and reagents. HeLa cells were cultured in DMEM (Media Tech) with 10% Fetal Bovine Serum (Hyclone) and 1% Penicillin Streptomycin (Invitrogen). HeLa cells stably expressing M4-blast^R constructs were established by lentivirus infection followed by selection with 4 μg/ml Blasticidin (Invitrogen).

Expression constructs. All RIN1 expression constructs were made in lentivirus vectors. RIN1 wild type, RIN1^{E574A} were made in in pM4-blast^R vector.[23] In RIN1^{QM}, Tyrosines 36, 121, 148, and 295 were mutated to phenyalanine. The RIN1^{QM} construct was made in the M4-IRES-GFP vector.[24] Virus production and transduction were performed as previously described.[8]

The RAB5-GTP pulldown construct was created using the Zn^{2+} finger domain of rabenosyn (ZFYVE20), a RAB5 binding domain. The sequence encoding amino acids 1–40 of human ZFYVE20 was amplified using forward primer 5'-ATGCGCTAGC AGATCTACTA GTATGGCTTC TCTGGACGAC CC and reverse primer 5'-ATGCGGATCC TTATCTAGAT TCCCCTGAGT GTTCTTCCT. Ligation compatible restriction sites in each primer (XbaI/SpeI and BamHI/BglII) were used for stepwise head-to-tail additions in pKS bluescript. The 4x concatemer was then inserted into the BamHI-EcoRI sites of pGEX-2T to create pGEX-4xZFYVE (R5BD-GST).

RAB5 pull-down. Active RAB5 from cells were pulled down using the construct: pGEX-4XZFYVE-GST, purified on glutathione beads as previously described.[9] Briefly, 1 × 10^6 HeLa cells were seeded overnight per condition. Cells were serum starved for about 18 h. Stimulation was performed with control medium or 100 ng/ml EGF medium. Cells were lysed after the respective times with NP-40 lysis buffer (150 mM Tris pH 8.0, 50 mM NaCl, 1% NP-40) containing 1 mM PMSF, 10 ug/ml Leupeptin, 1 uM Pepstatin and 1 mM Sodium Orthovanadate (Sigma Aldrich) in the presence of 10 mM $MgCl_2$. Lysates were subject to pull-down at 4°C with 4X-ZFYVE-GST on Glutathione beads in order to enrich for activated RAB5. Beads were then washed with the lysis buffer, boiled in Laemmli loading buffer, followed by gel electrophoresis and analysis of RAB5 by immunoblotting.

For immunoblotting, proteins were transferred to Nitrocellulose membranes overnight. The membranes were blocked with 5% milk in TBST (0.1% Tween-20) followed by incubation with primary and secondary antibodies at room temperature. The membranes were washed with TBST between the incubations and developed using the ECL plus western blotting reagent (VWR) or scanned using a Li-Cor Odyssey scanner. Quantification of immunoblots was performed using the Li-Cor Odyssey software or ImageJ (NIH). Antibodies used for immunoblotting and their sources were Pan-RAB5 1:1000 (Abcam, #ab18211), Tubulin 1:5000 (Sigma Aldrich, #T6074-200 ul), RIN1 1:1000 (Mouse mAb, clone #C9E11, Colicelli lab, AbPro), sheep-anti-mouse-HRP 1:3000 (Amersham Biosciences, #NA931), goat-anti-rabbit-HRP 1:3000 (Kirkegaard and Perry, #4741506), goat-anti-rabbit-IRDye 800 1:5000 (Li-Cor Biosciences, #926-32211) and goat-anti-mouse-IRDye 680 1:5000 (Li-Cor Biosciences, #926-32220).

Cell migration. 1×10^6 HeLa cells were seeded in 10 cm plates overnight in triplicate. Cells were serum starved for about 12 h. Boyden chambers (cell culture inserts, 8 μm, BD Falcon) were coated with 10 ug/ml Fibronectin overnight at 4°C. The cell culture inserts were rinsed once in PBS and placed on 24-well plates, containing either control medium or 100 ng/ml EGF medium. Cells were harvested and seeded at 10^5 cells per ml. Cells were allowed to migrate across the transwell at 37°C for 24 h. The chambers were then rinsed in PBS and fixed with 4% PFA. Cells that had migrated were stained with crystal violet and counted under the microscope using a hemocytometer.

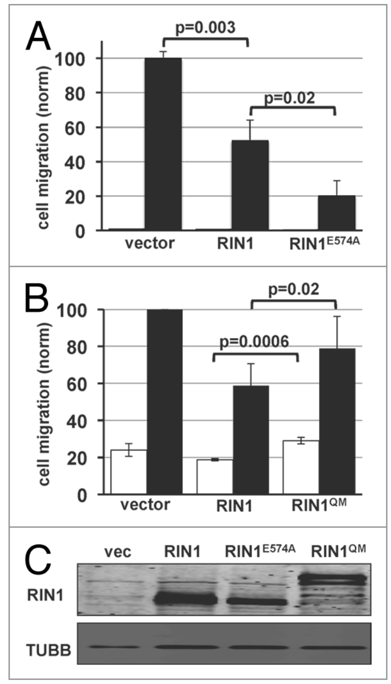

Figure 2. RIN1→RAB5 signaling axis promotes directional migration, while the RIN1→ABL axis blocks cell migration. (**A**) Migration across a transwell over one day toward control medium (white bar) or 100 ng/ml EGF medium (black bar) for vector, RIN1 and RIN1^{E574A} HeLa cells. (**B**) Migration across a transwell, as in (**A**), for vector, RIN1 and RIN1^{QM} HeLa cells. (**C**) Immunoblot showing RIN1 protein levels in vector, RIN1, RIN1^{E574A} and RIN1^{QM} HeLa cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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