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海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。

なお、下記及び別紙記載の内容については相違ありません。

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Phosphorylated Rho-GDP Directly Triggers mTORC2 Kinase Activity Toward AKT Through Heterotypic Dimerisation with Ras-GTP

Hiroshi Senoo

ABSTRACT

G-protein-coupled receptors (GPCRs) activate mTORC2-AKT signalling in metabolism, cell survival and cytoskeleton dynamics. Altered mTORC2-AKT signalling leads to many human diseases, including cancer, metastasis and metabolic syndromes; however, it is unknown how mTORC2 is activated downstream of GPCRs. Using proteomic approaches, I identified a GDP-bound RhoGTPase as an mTORC2-binding protein and identified its function in mTORC2-AKT signalling by reconstituting GPCR-mediated mTORC2-AKT activation with purified mTORC2, Rho and Ras. How could GDP-bound Rho function as an activator for mTORC2? I identified that glycogen synthase kinase-3 (GSK-3) directly phosphorylates GDP-bound Rho at a specific serine residue in response to GPCR stimulation. This phosphorylation enables GDP-bound Rho to assemble a signalling super-complex with mTORC2 and GTP-bound Ras to promote robust AKT phosphorylation¹. (Senoo et al., 2019, *Nature Cell Biology*, In Press)

Mutations that lock human Rho in a GDP-bound form are driver mutations in lymphoma^{2,3}. In cancer cells carrying these Rho mutants, AKT phosphorylation and cell migration rates are dramatically increased. These findings have been explained by the sequestration of Guanine nucleotide Exchange Factors (GEF) through stabilised interactions with GDP-bound mutant. In contrast, my findings provide a new framework to understand the action of these prevalent cancer-associated Rho mutations. I believe that GDP-bound Rho directly activates the kinase activity of mTORC2 toward AKT and thereby promotes cancer cell proliferation and metastasis.

My discoveries rewrite the central dogma that G proteins are only active in a GTP-bound state and further our understanding of mTORC2-AKT-related human diseases.

1. Directed cell migration - the process by which cells migrate towards chemical gradient

Cells sense extracellular chemical gradients through receptors on the plasma membrane and convert this into intracellular signals. This chemotactic signalling is involved in many biological processes including the immune response, wound healing and cancer metastasis⁴⁻⁷, which are essential for us to maintain our bodily health. For example, when bacteria invade our bodies, neutrophils sense the chemical gradient secreted from bacteria through G-protein-coupled receptors (GPCRs). Next, in order for neutrophils to chase the bacteria, intracellular signalling which is required for chasing bacteria is locally activated towards the high concentration of the chemical gradient⁸ (Fig. 1A & B).

Mechanistic Target of Rapamycin (mTOR) kinase forms two separate complexes, mTORC1 and mTORC2. These two kinase complexes control distinct biological processes. While mTORC1 functions as a nutrient sensor and controls protein synthesis and cell growth, mTORC2 controls actin cytoskeletal dynamics by modulating the phosphorylation of AKT⁹⁻¹¹. During chemotactic cell migration, localised activation of RhoGTPases and mTORC2 at the leading edge of the migrating cells is required for chemotaxis. These signalling pathways stimulate the actin cytoskeleton and allow cells to extend pseudopods¹²⁻¹⁴. RhoGTPases are molecular switches; it is popularly believed that most of the RhoGTPases cycle from an “ON” GTP-bound state to an “OFF” or “Resting” GDP-bound state¹⁵⁻¹⁸.

Alterations in directed cell migration have been linked to many human diseases, including chronic inflammatory disease and tumour metastasis^{17, 19, 20}. Despite the importance of RhoGTPases and the mTORC2-AKT signalling pathway in cytoskeletal dynamics, we still have an incomplete understanding of these proteins. How or where does “Cross-Talk” between these two major proteins occur? In contrast to the prevailing view that GDP-bound G proteins are “inactive”, my study reveals that the mTORC2-AKT signalling pathway is activated by Rho-GDP.

2. GDP-bound RacE is sufficient to drive chemotaxis

The mechanisms underlying directed cell migration have been extensively studied in the social amoebae, *Dictyostelium*, as a model system. Subsequent studies have demonstrated that the signalling mechanisms characterised in *Dictyostelium* are highly conserved in human cells^{21, 22}. To understand which RhoGTPases control directed cell migration, I executed global gene disruption and followed up with phenotypic analysis. As a result, I found that the *Dictyostelium* RhoA homologue, RacE, is required for chemotaxis²³⁻²⁵. Interestingly, when I ectopically expressed WT-, GDP-, and GTP-bound RacE in RacE-KO cells, GDP-bound RacE restored the chemotaxis failure in RacE-KO cells to a similar extent as WT-RacE; in contrast, GTP-bound RacE failed to do so (Fig. 1C).

3. GDP-bound RacE binds and activates TORC2

To determine how RacE controls the chemotactic signalling pathway, I searched for RacE binding proteins using a combination of immunoprecipitation and mass-spectrometry. I identified two components of the mTORC2 signalling pathway: the *Dictyostelium* mTOR homologue, TOR, and its rictor homologue, PiaA²⁶. Subsequent co-immunoprecipitation revealed that WT- and GDP-bound RacE, but not GTP-bound, interacted with TOR and PiaA (Fig. 1D). To determine the function of RacE in the TORC2 signalling pathway, I examined AKT phosphorylation by TORC2 as a readout for TORC2 activation^{26, 27}. TORC2 phosphorylated AKT proteins in response to GPCR activation by chemoattractant stimulation in WT cells; however, this did not occur in RacE-KO cells, suggesting that RacE is required for TORC2 activation. Intriguingly, when I ectopically expressed WT-, GDP- and GTP-bound RacE in RacE-KO cells, WT- and GDP-bound RacE, but not GTP-bound, restored this TORC2-mediated AKT phosphorylation.

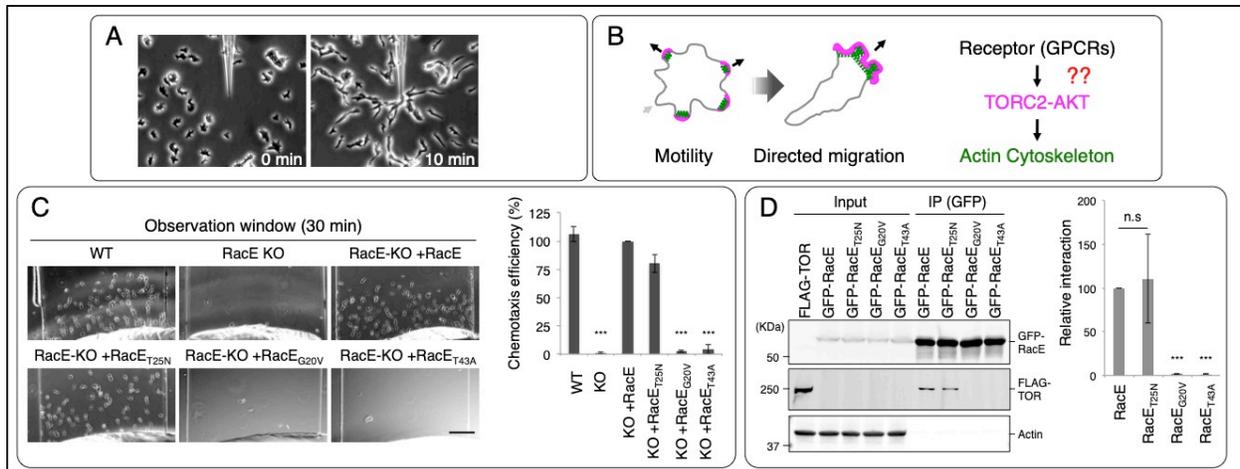


Fig. 1. Chemotactic response in *D. discoideum*. (A) Images were captured before (0 min) and after (10 min) a chemoattractant-filled micropipette was positioned in a field of cells. (B) Receptor (GPCRs) activation organises random motility into directed cell migration and allows cells to polarise the TORC2-AKT signalling pathway to remodel the actin-cytoskeleton. Pink indicates localised activation of TORC2-AKT signalling pathway. (C) Cell migration towards chemoattractant was analysed in the microfluidic chamber. Chemotaxis efficiency was determined by measuring the number of cells that moved toward the higher concentration of chemoattractant. Cells harboring WT-RacE and RacE^{T25N} (GDP-bound), RacE^{G20V} (GTP-bound) or effector domain-defective RacE^{T43A} in the microfluidic chamber. WT-RacE and RacE^{T25N} (GDP-bound) restores chemotaxis failure in RacE-KO cells. (D) Cell lysates harboring GFP fused to the indicated forms of RacE and FLAG-TOR were subjected to immunoprecipitation with GFP-Trap beads. WT-RacE and RacE^{T25N} (GDP-bound) strongly bind to TOR. Quantification of the band intensity of FLAG-TOR in immuno-precipitates.

It has been reported that RasGTPases, including the *Dictyostelium* K-Ras homologue RasC, play a critical role of TORC2-mediated AKT phosphorylation²⁶. AKT phosphorylation requires RasC and is increased by the ectopic expression of GTP-bound RasC; however, this impact is dramatically reduced in RacE-KO cells. These results suggest that GDP-bound RacE and GTP-bound RasC cooperate with each other to activate the TORC2-AKT signalling pathway.

4. Chemoattractant-induced phosphorylation regulates GDP-bound RacE

Many of the small GTPases are regulated by a GDP/GTP cycle in response to chemoattractant stimulation in which GTP binding activates them and the downstream signalling pathway¹⁵⁻¹⁷. However, as I mentioned earlier, RacE is different from a typical RhoGTPase in that the GDP-bound form activates downstream targets. Does RacE accommodate GDP in response to GPCR activation by chemoattractant stimulation? Consistent with the function of RacE-GDP and RasC-GTP in the chemotactic signalling pathway, as I expected metabolic labelling with P³² exhibited that GDP binding to RacE and GTP binding to RasC significantly increased in response to GPCR activation by chemoattractant stimulation.

The combination of immunoprecipitation and quantitative phospho-proteomics suggests that chemoattractant stimulation induces RacE phosphorylation at serine 192 in *Dictyostelium* cells. This phosphorylation motif is highly conserved at serine 188 in mammalian RhoA. I tested whether this phosphorylation regulates the function of RacE in the chemoattractant-induced activation of TORC2. When I ectopically expressed phospho-defective RacE_{S192A} or phospho-mimetic RacE_{S192E} in RacE-KO cells, I found that RacE_{S192A} failed to restore AKT phosphorylation in response to GPCR activation by chemoattractant stimulation; conversely, RacE_{S192E} not only restored but also enhanced AKT phosphorylation, even without chemoattractant stimulation, suggesting that chemoattractant-induced phosphorylation of RacE at serine 192 controls TORC2-mediated AKT phosphorylation.

5. GSK-3 phosphorylates RacE at serine 192 in response to chemoattractant stimulation

As I mentioned earlier, the combination of immunoprecipitation and quantitative phospho-proteomics showed that chemoattractant stimulation induces RacE phosphorylation at serine 192 and regulates the TORC2-AKT signalling pathway. To validate this phosphorylation, I successfully generated antibodies to phospho-RacE at serine 192. Using these antibodies, I found that RacE indeed undergoes transient phosphorylation upon chemoattractant stimulation (Fig. 2A, 15-60 sec). The kinetics of phosphorylation is identical to that of AKT, suggesting that RacE phosphorylation links the TORC2-AKT signalling pathway.

What kinase phosphorylates RacE in response to GPCR activation by chemoattractant stimulation? Since RacE phosphorylation at serine 192 is an essential step for TORC2 activation, the molecular identity of the RacE kinase is another major unanswered question; I executed a combination of proteomic, genetic, and biochemical approaches to address this question. To identify a protein kinase that phosphorylates RacE in response to GPCR activation by chemoattractant stimulation, I first analysed the amino acid sequence around serine 192 and found a glycogen synthase kinase-3 (GSK-3) substrate motif. GSK-3 is required for AKT phosphorylation in response to GPCR activation by chemoattractant stimulation²⁸. To determine whether RacE phosphorylation at serine 192 depends on GSK-3 in cells, I pre-treated *Dictyostelium* cells with LY2090314 and lithium, two structurally distinct, specific inhibitors of GSK-3, and stimulated these cells with the chemoattractant. I found that the chemoattractant-induced serine 192 phosphorylation of RacE was completely blocked. To validate this specific GSK-3 activity, I examined RacE phosphorylation at serine 192 in GSK-3-KO cells and found that chemoattractant-induced RacE phosphorylation was not detected. To determine if GSK-3 directly phosphorylates RacE at serine 192, I incubated purified human GSK-3 β with purified FLAG-tagged

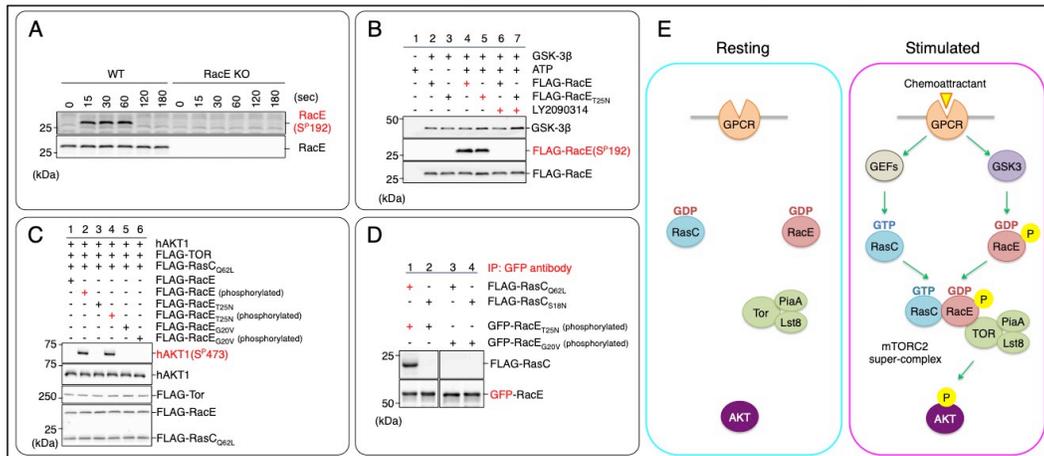


Fig. 2. (A) GSK-3 phosphorylates RacE at serine 192 in response to GPCR activation by chemoattractant. Total amounts of RacE and its phosphorylation at serine 192 were analysed by immunoblotting with antibodies to RacE and phospho-RacE (S192). (B) GSK-3 β directly phosphorylates RacE at serine 192 *in vitro*. WT-RacE or RacE_{T25N} (GDP-bound) was mixed with GSK-3 β in the presence or absence of the GSK-3 β inhibitor LY2090314 and examined for serine 192 phosphorylation. (C) Phosphorylated RacE (GDP-bound) activates TORC2 *in vitro*. TORC2-mediated AKT phosphorylation was reconstituted using purified proteins. TORC2 (FLAG-TOR), FLAG-RacE and FLAG-RasC were purified from *Dictyostelium* cells. Phosphorylated indicates that cells were stimulated by the chemoattractant before purification of FLAG-RacE. WT-RacE or RacE_{T25N} (GDP-bound), but not RacE_{S192A} (GTP-bound), activates TORC2 after the chemoattractant stimulation. (D) Phosphorylated RacE_{T25N} (GDP-bound) forms a GTPase dimer with RasC_{Q62L} (GTP-bound), but not RasC_{T18N} (GDP-bound). GFP-RacE was incubated with FLAG-RasC and pulled down using GFP-Trap. (E) Model for GPCR-mediated TORC2-AKT signalling pathway. RacE-GDP becomes phosphorylated by GSK-3 and assembles the super-signalling complex with Ras-GTP and TORC2 to promote AKT phosphorylation.

RacE (WT), RacE_{T25N} (GDP-bound) or phospho-defective RacE_{S192A} from *Dictyostelium* cells. I found that GSK-3 β phosphorylates RacE and RacE_{T25N}, but not RacE_{S192A}, at serine 192 *in vitro*. This *in vitro* phosphorylation of RacE by purified GSK-3 β was inhibited by the GSK-3 inhibitor LY2090314 (Fig. 2B), suggesting that GSK-3 directly phosphorylates serine 192 in RacE in response to GPCR activation by chemoattractant stimulation.

6. Reconstitution of GDP-bound RacE-regulated TORC2 activation *in vitro*

As I mentioned earlier, GDP-bound RacE and GTP-bound RasC cooperate with each other to activate the TORC2-AKT signalling pathway. To elucidate the molecular mechanism by which GDP-bound RacE regulates TORC2, I biochemically reconstituted the chemoattractant-induced activation of TORC2 using proteins purified from *Dictyostelium* cells. I individually immunopurified each of the following FLAG-tagged proteins: 1. TOR, 2. WT-RacE, 3. RacE_{T25N} (GDP-bound) and 4. RasC_{Q62L} (GTP-bound). The RacE proteins were purified from *Dictyostelium* cells that were stimulated by the chemoattractant. I used inactive human AKT as a substrate and detected its phosphorylation using anti-phospho AKT antibodies (serine 473)^{29,30}. I found that AKT was robustly phosphorylated when incubated with TORC2, chemoattractant stimulated RacE (WT- or GDP-bound), and RasC_{Q62L} (GTP-bound) (Fig. 2C, lane 2 & 4). However, AKT phosphorylation was not observed when the RacE proteins were purified from unstimulated cells. Unlike WT- and GDP-bound RacE, GTP-bound RacE did not promote AKT phosphorylation regardless of the stimulation (Fig. 2C, lane 6). Further demonstrating the role of the chemoattractant stimulation, I found that this stimulation is bypassed when phospho-mimetic mutations S192E is introduced into WT- or GTP-bound RacE. Thus, I discovered a novel principle that phosphorylation of GDP-bound G-proteins produces an active signalling molecule in cells and *in vitro*. This new concept is awarded to deliver a paradigm shift in biology and medicine.

7. Serine 192 phosphorylation of RacE-GDP assembles the RasC-RacE-TORC2 complex

Previous studies have shown that the localisation of mTORC1 is regulated by smallGTPases called Rags. A Rag dimer formed by Raga/B-GDP and RagC/D-GTP is anchored to the lysosomal membrane through the Regulator protein; however, Rag dimers themselves are not necessary for the enzymatic activity of mTORC1¹⁰. It has been reported that GTP-bound RasC regulates the localisation and activity of TORC2 on the plasma membrane²⁶. How does phosphorylated GDP-bound RacE stimulate TORC2? To understand the activation mechanism, it is essential to elucidate how RacE, RasC and TORC2 interact with one another. Therefore, I initiated an *in vitro* interaction analysis using purified proteins from *Dictyostelium* cells, 1. TOR, 2. RacE_{T25N} (GDP-bound and purified from cells that were chemoattractant-stimulated) and 3. RasC_{Q62L} (GTP-bound) and found that chemoattractant stimulated RacE_{T25N} directly interacts with TOR. In contrast, I did not see direct interactions between TOR and RasC_{Q62L}. Instead, RasC_{Q62L} interacted with RacE_{T25N} that was purified from chemoattractant stimulated cells (Fig. 2D, lane 1). Therefore, I concluded that phosphorylated, GDP-bound RacE forms a GTPase dimer with GTP-bound RasC to activate TORC2 (Fig. 2E, right).

8. Perspective: GDP-bound RacE is a potential therapeutic target of AITL

It has been suggested that the RhoA mutation encoding G17V is a cancer driver mutation in AngioImmunoblastic T-cell Lymphoma (AITL), which is a common type of mature T-cell lymphoma with poor prognosis^{31, 32}. Therefore, functional analysis of RhoA_{G17V} has potential implications for developing therapeutic targets for a large proportion of T-cell lymphomas. I have found that a *Dictyostelium* RacE_{G23V} mutation that corresponds to RhoA_{G17V} and that RacE_{G23V} robustly increases TORC2-mediated AKT phosphorylation *in vivo* and in my *in vitro* system. Therefore, there are common features between GDP-bound *Dictyostelium* RacE and human RhoA, e.g., similar to GDP-bound RacE, GDP-bound RhoA, not only increases mTORC2-mediated AKT phosphorylation, but also enhances actin cytoskeleton-based cell migration. In summary, taking together my discovery that GDP-bound RhoGTPase is an activator might bridge developing therapeutic targets for molecular pathogenetic insight into AITL.