The small GTPases Ras and Rap1 bind to and control TORC2 activity

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Target of Rapamycin Complex 2 (TORC2) has conserved roles in regulating cytoskeleton dynamics and cell migration and has been linked to cancer metastasis. However, little is known about the mechanisms regulating TORC2 activity and function in any system. In Dictyostelium, TORC2 functions at the front of migrating cells downstream of the Ras protein RasC, controlling F-actin dynamics and cAMP production. Here, we report the identification of the small GTPase Rap1 as a conserved binding partner of the TORC2 component RIP3/SIN1, and that Rap1 positively regulates the RasC-mediated activation of TORC2 in Dictyostelium. Moreover, we show that active RasC binds to the catalytic domain of TOR, suggesting a mechanism of TORC2 activation that is similar to Rheb activation of TOR complex 1. Dual Ras/Rap1 regulation of TORC2 may allow for integration of Ras and Rap1 signaling pathways in directed cell migration.

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Results

**Rap1 is a conserved binding partner of TORC2 component RIP3/SIN1 whereas RasC binds TOR.** To identify proteins regulating TORC2 function, we expressed His/Flag-tagged Pianissimo (HF-Pia), the Dictyostelium orthologue of mammalian TORC2 essential component Rictor, in *piaA* null cells, and used HF-Pia to purify TORC2 from cells stimulated by the chemoattractant (Fig. 1a). Proteins that co-purify with HF-Pia were identified by mass spectrometry. As expected, known components of TORC2, and of protein synthesis and folding complexes co-purify with HF-Pia (see Supplementary Table S1). Of interest, we found that the small GTPase RasP was specifically pulled-down with HF-Pia (Fig. 1b). In addition, in a pull-down screen that we previously performed with recombinant, purified GST-fused Rap1 pre-loaded with non-hydrolyzable GppNHp (active state) or GDP (inactive state)\(^1\), we found that the TORC2 component RIP3 (orthologue of mammalian SIN1) specifically co-purifies with Rap1\(^{\text{GppNHp}}\) (Fig. 1c). Together with our finding that Rap1 co-purifies with Pia, this observation suggests that Rap1 interacts with TORC2 by directly binding RIP3.

Similar to most Ras/Rap effectors, RIP3/SIN1 contains a Ras Binding Domain (RBD)\(^2\).\(^3\).\(^4\).\(^5\).\(^6\).\(^7\).\(^8\).\(^9\).\(^10\).\(^11\).\(^12\) Despite low amino acid sequence conservation, all RBDS have a typical ubiquitin-like fold that facilitates binding to Ras proteins. The RBD of RIP3 was previously shown to be important for TORC2 function in *Dictyostelium* chemotaxis and to bind the active form of the Ras protein RasG, and not RasC, *in vitro*.\(^11\)\(^12\).\(^13\) However, *in vivo*, RasC, and not RasG, promotes TORC2 activation\(^14\).\(^15\).\(^16\).\(^17\).\(^18\) To determine if Rap1 directly interacts with the RBD of RIP3 (RIP3\(^{\text{RBD}}\)), we assessed their binding *in vitro*, compared to the binding of RIP3\(^{\text{RBD}}\) to other Dictyostelium Ras proteins (RasB, RasC, RasD, RasG and RasS). We found that only the active forms of Rap1 and RasG bind RIP3\(^{\text{RBD}}\) *in vitro* (Fig. 1d). These results suggest that RasC activates TORC2 by directly regulating TOR kinase.

**Activation of TORC2 by RasC**

Although others and we have clear evidence that RasC promotes TORC2 activation in response to chemotactic stimulation in *Dictyostelium* (present study)\(^1\)\(^2\)\(^3\)\(^4\), we find that, unlike Rap1, RasC does not bind RIP3\(^\text{RBD}\) (Fig. 1d). However, a constitutively active RasC mutant (RasC\(^{\text{C35L}}\)) specifically co-purifies with RasC\(^{\text{GppNHp}}\) (Fig. 1g). We verified that RasC binds directly to TOR using recombinant, purified proteins *in vitro*. We observed that wild-type cell lysates induce PKB (Akt) T435 phosphorylation in *piaA* null cells, and used PKB (Akt) to purify TORC2 from cells stimulated by the chemoattractant (Fig. 1a). Proteins that co-purify with PKB (Akt) were identified by mass spectrometry. As expected, known components of TORC2, and of protein synthesis and folding complexes co-purify with PKB (Akt) (see Supplementary Table S1). Of interest, we found that the small GTPase Rap1 was specifically pulled-down with PKB (Akt) (Fig. 1b). In addition, in a pull-down screen that we previously performed with recombinant, purified GST-fused Rap1 pre-loaded with non-hydrolyzable GppNHp (active state) or GDP (inactive state)\(^1\), we found that the TORC2 component RIP3 (orthologue of mammalian SIN1) specifically co-purifies with Rap1\(^{\text{GppNHp}}\) (Fig. 1c). Together with our finding that Rap1 co-purifies with Pia, this observation suggests that Rap1 interacts with TORC2 by directly binding RIP3.

**Rap1 regulates the RasC-mediated activation of TORC2.** To assess whether Rap1, in addition to RasC, can induce TORC2 activation, we first examined the ability of purified, recombinant Rap1, loaded with GppNHp, to induce TORC2 activation in cell lysates. We monitored TORC2 activation by evaluating its phosphorylation of Akt/Protein Kinase B (PKB) and related kinase PKBR1 hydrophobic motif (TP435 and TP470, respectively)\(^1\)\(^2\)\(^3\)\(^4\).\(^5\)\(^6\). Consistent with previous findings\(^1\)\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\), RasC\(^{\text{GppNHp}}\) stimulation of wild-type cell lysates induces the TORC2-mediated phosphorylation of both PKB (~50 kDa band) and PKBR1 (~70 kDa band) (Fig. 2). Interestingly, we found that Rap1\(^{\text{GppNHp}}\) also stimulates PKB T435 and PKBR1 T470 phosphorylation (Fig. 2). This suggests that Rap1 is functional in the wild-type cells, whereas RasC is not. However, RasC\(^{\text{GppNHp}}\) and Rap1\(^{\text{GppNHp}}\) do not induce PKB phosphorylation in *piaA* null cell lysates (see Supplementary Fig. S4), confirming that the observed responses are mediated by TORC2. Further, Rap1\(^{\text{GppNHp}}\), RasC\(^{\text{GppNHp}}\), and Rap1\(^{\text{GDP}}\) fail to promote TORC2 activation in wild-type cell lysates, indicating that only active RasC and Rap1, and not RasG, can promote TORC2 activation.

As cells lacking Rap1 are not viable, to test whether Rap1 controls TORC2 activity *in vivo*, we compared the chemoattractant-induced TORC2 activation in wild-type cells to that in cells displaying elevated Rap1 activity either by overexpressing wild-type Rap1 (Rap1\(^{\text{OE}}\)) or by expressing a constitutively active Rap1 mutant (Rap1\(^{\text{CA}}\), G12V mutation), or in cells lacking one of the Rap1-specific GAP, RapGAP1 (rapgap1 null), in which Rap1 activity is considerably elevated\(^1\)\(^2\)\(^4\)\(^2\)\(^4\) (see Supplementary Fig. S6a). In this assay, PKB T435 phosphorylation is sometimes difficult to detect, but PKB phosphorylation is easily traced. As shown in Fig. 3a, we reproducibly observed elevated and extended chemoattractant-induced PKB phosphorylation in all three conditions tested.
Figure 1. TORC2 binds RasC and Rap1 through different complex components. (a) Developed piaA null cells expressing HF-Pia were stimulated with the chemoattractant (10 μM cAMP) for 10 sec, followed by sequential His-Flag purification. Proteins pulled-down with HF-Pia were resolved by SDS-PAGE and stained with silver. Wild-type cells were used as control. (b) Mass spectrometry data identifying Rap1 co-purifying with HF-Pia. The full list of proteins identified in the HF-Pia pull-down is available in Supplementary Table S1. (c) Mass spectrometry data identifying RIP3 in the pull-down performed with GST-Rap1<sub>GppNHp</sub>. (d) Interaction between GST-RIP3<sup>RBD</sup> and His-tagged Rap1, RasG, RasB, RasC, RasD, and RasS was assessed using recombinant, purified proteins in vitro, comparing the binding of constitutively active (CA) and GDP-bound (inactive) Rap1/Ras proteins. His-Rap1/Ras proteins were detected by immunoblotting. Immunoblots were cropped, but no other bands were present. Amount of Ras/Rap1 proteins used is shown in Supplementary Fig. S1a. (e) Dissociation of mGppNHp from Rap1 and RasG was measured in the presence and absence of 1 μM RIP3<sup>RBD</sup> or RIP3<sup>RBD</sup>, His peptide, GppNHp, or EDTA human Rap1b was assessed using recombinant, purified proteins in vitro. His-peptide was used as control. Loadings are equivalent between the input and His-Pull-down conditions. Band at ~24 kDa in GST-SIN1<sup>RBD</sup> input corresponds to free GST. Pull-down proteins were revealed by Commassie Blue (CB) staining. (g) Mass spectrometry data identifying TOR in the pull-down performed with GST-RasC<sup>GppNHp</sup>. (h) The interaction between GFP-fused TOR catalytic domain (FRB/Kin<sup>TOR</sup>) and GST-fused GppNHp- or GDP-bound RasC was assessed using recombinant, purified proteins in vitro. GFP-FRB/Kin<sup>TOR</sup> and GST-RasC were revealed by immunoblotting. Input represent 33% of protein used in assay. Uncropped gel and immunoblots are shown in Supplementary Fig. S2. Data are representative of at least two independent experiments.
where Rap1 activity is elevated compared to that in wild-type cells. Although the extent of the effect of Rap1CA on PKBR1 phosphorylation varied between experiments, likely due to varying levels of Rap1 CA expression related to plasmid copy number per cell as was previously described25–27, quantification of the data nonetheless revealed significant (at 40 and 60 sec) or near-significant effects (5 sec, $p = 0.053$; 10 sec, $p = 0.084$; 20 sec, $p = 0.077$). For Rap1OE and rapgap1 null cells, the effects are strongly significant (for 10 and 20 sec time points, $p$ is between 0.0003 and 0.004). Consistent with an increase in TORC2-mediated PKBR1 phosphorylation, PKBR1 kinase activity is also prolonged in Rap1CA, Rap1OE, and rapgap1 null cells, although the effect is not as pronounced as that on PKBR1 T470 phosphorylation (Fig. 3b). This difference is likely due to the fact that the PKB kinase activity is not only regulated by TORC2 but also by other chemotactic effectors and regulatory mechanisms (e.g. GSK3, Protein Phosphatase 2A28,29).

The increase in PKBR1 phosphorylation observed in rapgap1 null, and in Rap1CA and Rap1OE cells is not observed in rapgap1/ripA double null cells or ripA null cells expressing Rap1 CA or Rap1OE, nor is there any TORC2-mediated PKBR1 phosphorylation observed in ripA null cells expressing the RIP3 (K680E,R681E) mutant (Fig. 3c). These observations indicate that Rap1 interaction with RIP3 is necessary for the observed effect of elevated Rap1 activity on TORC2-mediated PKBR1 phosphorylation and, thus, that Rap1 plays an important role in controlling TORC2 activation. Of note, we sometimes detect constitutive PKB phosphorylation in ripA null cells (Fig. 3c and Supplementary Fig. S6b), as was previously observed7, but the meaning of which is unknown. Interestingly, however, neither Rap1OE nor Rap1CA induce PKBR1 phosphorylation in cells lacking RasC, suggesting that RasC is essential for TORC2 activation and the Rap1-mediated effect on TORC2 (Fig. 3d). Consequently, we propose that Rap1 positively regulates the RasC-mediated activation of TORC2 in response to chemoattractants.

Discussion
Our findings reveal new, and likely conserved, mechanisms by which Dictyostelium TORC2 is regulated, where both RasC and Rap1 control TORC2 activity through binding of distinct TORC2 components, TOR and RIP3/SIN1, respectively (Fig. 4). In addition, our findings suggest that RasC plays a major role in TORC2 activation and that Rap1 regulates the RasC-mediated activation of TORC2. Whereas we can’t exclude the possibility that Rap1 binds RIP3/SIN1 independently of TORC2, the observation that Rap1 co-purifies with the TORC2 component Pia/Rictor strongly suggests Rap1 binds the TORC2-associated RIP3/SIN1 and, thereby, directly interacts with TORC2. The finding that RasC binds to the catalytic domain of TOR is particularly interesting as it indicates that RasC may activate TORC2 using a mechanism that is similar to the reported Rheb-mediated activation of mTORC119, and that RasC may regulate TORC1 as well. Moreover, as human H-Ras was reported to co-purify with mTORC221, we believe that Ras binding to TOR is likely conserved in mammals. Unfortunately, we were unable to test direct binding of human H-Ras to mTOR in vitro due to the difficulty to obtain stable, recombinant mTOR constructs.

Other proteins that we found associated with Dictyostelium TORC2 that are known to or could play a role in regulating TORC2 function in chemotaxis include Rac1A and Rab small GTPases, as well as the actin nucleator Formin A. TORC2 interaction with Formin A could represent a potential link between TORC2 and F-actin. In mammals, Rac1 binds TOR and mediates TORC1 and TORC2 localization to specific membranes86, and in fission yeast, a Rab-family GTpase was shown to bind and regulate TORC2 signaling31. Therefore, our finding that Rac1A and two Rab GTPases, Rab11A and RabC, bind TORC2 suggests that the role of these small GTPases in regulating TORC2 may be conserved in Dictyostelium. Finally, although the identification of ribosomal proteins in our HF-Pia pull-down is expected, as HF-Pia was exogenously over-expressed, it is possible that the binding of

Figure 2. Active RasC and Rap1 both induce TORC2 activation in cell lysates. Cell lysates were stimulated with recombinant, purified, GppNHp- or GDP-bound RasC, Rap1, and RasG for the indicated time. Phosphorylation of PKB (T435; ~50 kDa) and PKBR1 (T470; ~70 kDa) was detected by immunoblotting as a measure of TORC2 activation. Data are representative of at least two independent experiments. Uncropped immunoblots are shown in Supplementary Fig. S5.
Figure 3. Rap1 positively regulates the RasC-mediated activation of TORC2. (a,c,d) TORC2 phosphorylation of PKB (T383) and PKBRI (T367) was assessed in wild-type cells (WT), in cells lacking RapGAP1 (rapgap1−), RIP3 (ripA−), both RIP3 and RapGAP1 (rapgap1−ripA−), or RasC (rasC−), and in wild-type, ripA−, and rasC− cells expressing tagged Rap1CA or Rap1OE, and in ripA− cells expressing either wild-type T7-RIP3 or T7-RIP3(K680E,K681E). Cells were stimulated with 10 μM cAMP for the indicated time. PKB/ PKBRI phosphorylation and expression of exogenous Rap1, Rap1CA, RIP3, and RIP3(K680E,K681E) were revealed
kinase activity of immunoprecipitated PKBR1 was assessed in the indicated strains using H2B as substrate. H2B phosphorylation was detected by autoradiography and PKBR1 revealed by immunoblotting. Graphs represent mean ± SEM of densitometry quantification data of immunoblots or autoradiographs, from at least three independent experiments, expressed as % of the maximal signal detected in wild-type control cells. *p < 0.05, **p < 0.01, ***p < 0.005. Uncropped immunoblots and autoradiographs are shown in Supplementary Fig. S7.

by immunoblotting. Equal loading was controlled with Coomassie Blue staining (CB). (b) cAMP-induced kinase activity of immunoprecipitated PKBR1 was assessed in the indicated strains using H2B as substrate. H2B phosphorylation was detected by autoradiography and PKBR1 revealed by immunoblotting. Graphs represent mean ± SEM of densitometry quantification data of immunoblots or autoradiographs, from at least three independent experiments, expressed as % of the maximal signal detected in wild-type control cells. *p < 0.05, **p < 0.01, ***p < 0.005. Uncropped immunoblots and autoradiographs are shown in Supplementary Fig. S7.

We propose that the dual RasC- and Rap1-mediated regulation of TORC2 in Dictyostelium allows for integration of RasC and Rap1 signaling pathways during chemotaxis and, thereby, coordination of cytoskeletal remodeling, substrate adhesion, and relay of the chemoattractant signal to neighboring cells (Fig. 4). The organized migration of groups of cells is crucial to Dictyostelium and human embryonic development as well as to wound healing, and is also involved in cancer metastasis34–39. For cells to achieve collective migration as a cohesive group, they must synchronize their movement, which is achieved through cell-cell communication such as signal relay during chemotaxis. Our findings place TORC2 in an ideal position to promote the coordinated regulation of these processes and control group cell migration. Since the interactions between TORC2 and Ras/Rap1 appear conserved, we suggest TORC2 integrates these signals to coordinate cellular migrations in many systems.

Methods

Reagents and antibodies. cAMP sodium salt monohydrate was from Sigma-Aldrich (St. Louis, MO, USA) and H2B was from Roche-Genentech (San Francisco, CA, USA). Anti-myc (Myc A7) was from Abcam (Cambridge, MA, USA), anti-Pan Ras (Ab-3; RAS10) was from Calbiochem/EMD Millipore (Billerica, MA, USA), anti-GFP was from Covance (Princeton, NJ, USA), horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), and Dylight™ secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Anti-GFP, anti-GST, protein-A sepharose, and GSH affinity resin were from GE Healthcare (Pittsburgh, PA, USA). DdPKBR1 antibody was described previously40.

DNA constructs. His-Flag-Pianissimo (HF-Pia) was generated by adding 6XHis and Flag tags, in tandem, by PCR to the N-terminus of Pia’s coding sequence, which was cloned in the pDM304 vector containing a neomycin resistance cassette. Myc-tagged constitutively active Rap1 (Myc-Rap1CA; G12V mutation) obtained from Rick Firtel14 was transferred to the pDM358 vector containing a hygromycin resistance cassette. FRB/KinTOR (aa 1820–2380) was amplified by PCR and subsequently cloned in the pDM317 vector containing a terminal GFP and neomycin resistance cassette. The RIP3RBD (aa 648–717) used in the in vitro binding assay (Fig. 1d), and RasC, were expressed as N-terminal GST-fusion from a pGEX-4T-1 vector. Constitutively active RasC (RasC, G62L mutation) was generated by the method of Quick change. 6XHis-tagged wild-type and constitutively active forms of the Rap1 and Ras proteins were described previously41. The RIP3RBD (aa 511–838) and SIN1RBD (aa 266–374), used in the GDI experiment (Fig. 1e) and in vitro binding assay (Fig. 1f), respectively, were expressed as N-terminal GST-fusion from a pGEX-4T-3 plasmid. His-Rap1b was a kind gift of Alfred Wittinghofer.

Cell culture and strains used. Dictyostelium cells were grown in axenic HL5 medium (ForMedium, Hunstanton, Norfolk, UK) at 22 °C and transformants were generated by electroporation. Transformed cells were selected in 20 μg/ml Geneticin or 50 μg/ml Hygromycin B (both from Life Technologies, Grand Island, NY, USA) and expression confirmed by immunoblotting. Aggregation-competent cells were obtained by pulsing cells with 30 nM cAMP every 6 min for 5.5 h in 12 mM Na/K phosphate pH 6.1 at 5 × 10⁶ cells/ml. Wild-type cells are AX3 and all transformants and null strains have an AX3 background. gbpD null cells were described elsewhere42, piaA null and rapgap1 null cells were provided by Peter Devreotes and Rick Firtel, respectively, and were previously described24,25. The rapgap1/ripA double null strain was generated by disrupting ripA in the rapgap1 null background, as described previously41.

Pull-downs and mass spectrometry. Sequential His-Flag purification and identification of the isolated proteins by mass spectrometry was performed as previously described5. The pull-down screens for RasC and Rap1 effectors were performed as previously described16,44.

In vitro binding studies. GST-fused RIP3RBD, -SIN1RBD, -Rap1, and -RasC were purified by GSH affinity and size exclusion chromatography as previously described18,42. Purification of His-tagged Rap1 and Ras proteins, and the in vitro interaction assay with RIP3RBD was performed as described previously41. Purified proteins’ quality was verified on gel and quantified (see Supplemental Fig. S1), and equal amounts were used for each interaction assessed. His-Rap1/Ras proteins were detected by immunoblotting with anti-His monoclonal antibody (sc-8036; Santa Cruz Biotechnology, Dallas, TX, USA). Interaction between His-tagged GppNHp-bound or nucleotide free (EDTA) human Rap1b and GST-SIN1RBD was tested using 25 μg of the purified proteins incubated in binding buffer (50 mM Tris-Cl, 150 mM NaCl, 5 mM MgCl2, 1 mM β-mercaptoethanol, pH 7.5), and proteins were pulled-down with Ni-NTA affinity resin (Qiagen, Valencia, CA, USA) for 2 h at 4 °C. The beads were washed three times with ice-cold binding buffer containing 500 mM NaCl and eluted with 300 mM imidazole in binding buffer. The proteins were resolved on SDS-PAGE and revealed by Coomassie Blue staining. GFP-FRB/KinTOR was isolated from Dictyostelium cell lysates using anti-GFP antibody coupled to protein A Sepharose.
beads. Interaction between GFP-FRB/KinTOR and GST-fused GppNHp- or GDP-loaded RasC was tested using 25 μg of the purified proteins incubated in binding buffer (50 mM Tris-Cl, 150 mM NaCl, 5 mM MgCl₂, 1 mM β-mercaptoethanol, pH 7.5), and proteins were pulled-down with GSH affinity resin. The beads were washed three times with ice-cold binding buffer containing 500 mM NaCl, and eluted with 20 mM glutathione in binding buffer. The proteins were resolved on SDS-PAGE and revealed by GFP and GST immunoblotting.

**Biochemical assays.** The guanine nucleotide dissociation inhibition (GDI) assay, Rap1 activity assay, and PKBR1 kinase assay were performed as previously described 14,18,40. To test the ability of Rap1, RasC or RasG to activate TORC2 in cell lysates, the GTPases were loaded with GDP or GppNHp as previously described 18. Aggregation competent wild-type cells were harvested by centrifugation and re-suspended in buffer containing 50 mM Tris-Cl (pH = 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 5% Glycerol. Cells were lysed on 5 μm Nuclepore filter and the lysate was cleared by 16,000 x g centrifugation for 5 min at 4 °C. Total cell lysate protein content and purified Ras/Rap1 were quantified with Bradford reagent and 400 μg of cell lysate was stimulated with 1 μM of nucleotide bound GTPases and samples collected at the indicated times. 50 μg of proteins were loaded on gel for each sample. TORC2 activity was assessed by evaluating the TORC2-mediated phosphorylation of PKB and PKBR1 as described previously5, with the exception that an anti-phospho-p70S6K antibody (Cell Signaling Technology, Danvers, MA, USA) was used to detect phosphorylation of PKB/PKBR1’s hydrophobic motif (TP435 and TP470, respectively). Significance of the data was analyzed using unpaired T-Test.

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performed the experiments and analyzed the data. A.Ko. and P.G.C. designed and supervised the study, and wrote the manuscript; G.W., S.P.B. and P.J.M.V.H. supervised parts of the study. All authors reviewed the manuscript.

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Author Contributions

A.Kh. and P.L. contributed equally to the work. A.Kh., P.L., A.J.C., N.M.M., P.B., Z.S., H.P., A.Ko. and P.G.C. performed the experiments and analyzed the data. A.Ko. and P.G.C. designed and supervised the study, and wrote the manuscript; G.W., S.P.B. and P.J.M.V.H. supervised parts of the study. All authors reviewed the manuscript.
