Arg-78 of Nprl2 catalyzes GATOR1-stimulated GTP hydrolysis by the Rag GTPases

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mTOR complex 1 (mTORC1) is a major regulator of cell growth and proliferation that coordinates nutrient inputs with anabolic and catabolic processes. Amino acid signals are transmitted to mTORC1 through the Rag GTPases, which directly recruit mTORC1 onto the lysosomal surface, its site of activation. The Rag GTPase heterodimer has a unique architecture that consists of two GTPase subunits, RagA or RagB bound to RagC or RagD. Their nucleotide-loading states are strictly controlled by several lysosomal or cytosolic protein complexes that directly detect and transmit the amino acid signals. GATOR1 (GTPase-activating protein (GAP) activity toward Rags)-1, a negative regulator of the cytosolic branch of the nutrient-sensing pathway, comprises three subunits, Depdc5 (DEP domain–containing protein 5), Nprl2 (NPR2-like GATOR1 complex subunit), and Nprl3 (NPR3-like GATOR1 complex subunit), and is a GAP for RagA/B. GATOR1 binds the Rag GTPases via two modes: an inhibitory mode that holds the Rag GTPase heterodimer and has previously been captured by structural determination, and a GAP mode that stimulates GTP hydrolysis by RagA but remains structurally elusive. Here, using site-directed mutagenesis, GTP hydrolysis assays, coimmunoprecipitation experiments, and structural analysis, we probed the GAP mode and found that a critical residue on Nprl2, Arg-78, is the arginine finger that carries out GATOR1’s GAP function. Substitutions of this arginine residue render mTORC1 signaling insensitive to amino acid starvation and are found frequently in cancers such as glioblastoma. Our results reveal the biochemical bases of mTORC1 inactivation through the GATOR1 complex.

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2 The abbreviations used are: mTORC1, mTOR complex 1; GATOR1, GTPase-activating protein (GAP) activity toward Rags; Depdc5, DEP domain–containing protein 5; Nprl2, NPR2-like GATOR1 complex subunit; Nprl3, NPR3-like GATOR1 complex subunit; SHEN, steric hindrance for enhancement of nucleotidase activity; KICSTOR, KPTN, ITFG2, C12orf66, and SZT2-containing regulator of mTORC1; SEAC, Seh1-associated complex; RL, arginine-related loop; IP, immunoprecipitation; SUMO, small ubiquitin-like modifier; HA, hemagglutinin.
carries out the GAP activity for GATOR1. Further validation of this catalytic site revealed its evolutionary conservation and importance in switching off mTORC1 signaling upon amino acid starvation.

Results and discussion

Canonical GAPs activate GTP hydrolysis of their target GTPases by inserting either an arginine finger (19) or an asparagine thumb (20) into the nucleotide-binding pocket. The polar side chain disperses the charges built up during GTP hydrolysis and thus accelerates the reaction rate. Using a cell line in which individual subunits of GATOR1 were knocked out, we performed a screen for residues that could serve as the catalytic site. As suggested in our previous study, the catalytic residue likely resides within the Nprl2–Nprl3 dimer, so we focused on these two subunits. In Nprl2-knockout cells, mTORC1 is constitutively active as reflected by the high and equal phosphorylation levels of S6K1 (pThr-389-S6K1) in the absence or presence of amino acids (Fig. 1A, lanes 1 and 2). We identified surface-exposed and conserved arginine residues on Nprl2 and mutated individual arginines to an alanine and introduced the mutants back into the Nprl2-knockout cells. We reasoned that if an arginine residue participates in stimulating GTP hydrolysis by RagA, the corresponding Arg*3Ala mutant would behave differently from WT Nprl2 as a loss-of-function mutant. Expression in the Nprl2-knockout cells of most of the mutants we generated (e.g. R279A in Fig. 1A) restored the response to amino acids similarly to the expression of WT Nprl2. However, one particular mutant, R78A, failed to resensitize Nprl2-knockout cells: despite expressing R78A at a similar level as WT Nprl2, these cells remained largely resistant to amino acid starvation (Fig. 1A, lanes 5 and 6). This defect was not caused by an impairment of binding to other known regulators of the mTORC1 pathway as the Nprl2(R78A) mutant coimmunoprecipitates similar amounts of endogenous GATOR2 and KICSTOR components as WT Nprl2 (Fig. 1B).

We performed a similar mutation screen with arginine mutants of Nprl3 in Nprl3-knockout cells and found all the mutants we created behave similarly to WT Nprl3 (data not shown). Therefore, we selected Arg-78 of Nprl2 as a potential candidate for being the catalytic residue.

To validate the result in vitro, we overexpressed and purified WT and Nprl2(R78A)-containing GATOR1 from FreeStyle 293-F cells and directly measured their stimulatory effects on GTP hydrolysis by the Rag GTPases (Fig. 2A). WT GATOR1 robustly stimulated GTP hydrolysis by the Rag GTPases in a dose-dependent manner (Fig. 2, B and D), whereas GATOR1 carrying the Nprl2(R78A) mutation failed to do so (Fig. 2, C and D).
ACCELERATED COMMUNICATION: Arg-78 of Nprl2 is catalytic

This result is consistent with our suggestion that the Arg-78 residue on Nprl2 is the catalytic residue on GATOR1 that triggers GTP hydrolysis by the Rag GTPases (Fig. 2E).

To study the effect of the surrounding environment around Arg-78 on the stimulatory effect of GATOR1, we focused on two loops localized near Arg-78, RL1 and RL2 (arginine-related loop; Fig. 3A), as they likely provide binding sites for the Rag GTPases or modulate the catalytic pocket. Mutating RL1 to a GS linker of the same length (PTLG(17–21)GSGSG) completely abolished the inhibition of mTORC1 that normally occurs in response to amino acid starvation, whereas mutating RL2 had no observable effect (Fig. 3B). This result suggests that RL1 is necessary for the GAP function of GATOR1. To understand the underlying mechanism that leads to the defect caused by RL1 mutation, we first performed a communoprecipitation (co-IP) experiment to probe the interaction between Nprl2 and the Rag GTPases (Fig. 3C). To our surprise, mutant Nprl2(RL1), as well as mutants carrying single-point mutations within this loop, pulled down similar amounts of the Rag GTPases as WT Nprl2 (Fig. 3C), suggesting that the interaction between Nprl2 and the Rag GTPases remains largely intact and that RL1 is likely not the main binding site. However, when we carried out a stimulated GTP hydrolysis assay in vitro, we observed a strong defect of a mutant within RL1, G20S, which fails to catalyze GTP hydrolysis by RagA (Fig. 3D–F). This result could explain the failure of RL1 to restore amino acid sensitivity in Nprl2-knockout cells (Fig. 3B) as a defective GATOR1 could not suppress mTORC1 signaling in the absence of amino acids. Kinetic analysis revealed that the G20S mutant behaves differently than the R78A mutant: we measured a low $k_{\text{cat}}$ with a finite $K_m$ for the R78A mutant, but the G20S mutant failed to reach saturation (Fig. 3, E and F). This difference suggests that the two mutants impair the GAP function via different mechanisms: R78A abrogates the active site, whereas G20S likely modulates the catalytic pocket and mispositions Arg-78 to confer its defect.

In the yeast Saccharomyces cerevisiae, the SEAC complex has been shown to share similar functions with human GATOR1 and carries out a GAP activity toward the Gtr proteins, the homologs of the Rag GTPases (14). We aligned the sequence of human GATOR1 subunits with those from mouse, fruit fly, and yeast (Fig. 4, A–C). Interestingly, the protein sequence around Arg-78 of Nprl2, which is responsible for the GAP function, is very well conserved (Fig. 4, A and B). In contrast, the critical strip on Depdc5 that mediates the interaction with the Rag GTPases in the inhibitory mode, is not (Fig. 4C). Considering the sequence conservation around Arg-78 of Nprl2, we wondered whether human GATOR1 could potentially stimulate GTP hydrolysis of the yeast Rag complex. To test this hypothesis, we purified Gtr1p–Gtr2p of S. cerevisiae and used it as the substrate for human GATOR1. Satisfactorily, we observed a strong stimulation of GTP hydrolysis in the presence of WT human GATOR1 (Fig. 4D, blue line). Moreover, when we eliminated the inhibitory mode by introducing the Depdc5(Y775A) mutation, the GAP activity did not change as dramatically (Fig. 4D, red line), which suggests that the GAP function of human GATOR1 is conserved in yeast, whereas the inhibitory binding mode is not. The similar activities of WT GATOR1 and the Depdc5(Y775A) mutant toward Gtr1p–Gtr2p is in sharp contrast to the situation with the human Rag GTPases as in the latter case the inhibitory binding mode dominates WT GATOR1 behavior (Fig. 4E). In accordance with this result, when we plotted the degree of sequence conservation on the surface of GATOR1 (Fig. 4F), we observed the highest degree of conservation in the catalytic pocket of Nprl2, suggesting that this GAP mode is preserved along the evolutionary path.

Conclusion

GATOR1 is a major negative regulator for mTORC1. In the absence of amino acids, GATOR1 executes GAP activity to RagA and RagB and turns off mTORC1 signaling. In this study, we identified the molecular basis of the GAP function and found that Arg-78 of Nprl2 is the catalytic residue. Mutation of this arginine residue completely abolishes GAP function in vitro and in cells. Furthermore, cross-activity of human GATOR1 toward the yeast Gtr proteins suggests the evolutionary conservation of the GAP mode.
Experimental procedures

Chemicals were obtained from Sigma-Aldrich, and 32P-labeled GTP was from PerkinElmer Life Sciences. Antibodies were obtained from the following resources: rabbit anti-FLAG, Cell Signaling Technology, 2708; rabbit anti-HA, Bethyl Laboratories, A190-208A; Rabbit anti-myc: Cell Signaling Technology 5605; rabbit anti-pThr-389-S6K1, Cell Signaling Technology, 9205; rabbit anti-S6K1, Cell Signaling Technology, 2708; rabbit anti-Raptor, EMD Millipore, 09-217; rabbit anti-MIOS, Cell Signaling Technology, 13557; rabbit anti-WDR59, Cell Signaling Technology, 53385; rabbit anti-SZT2, Cell Signaling Technology, bleeds; rabbit anti-KPTN, Proteintech, 16094-1-AP; goat-anti-rabbit horseradish peroxidase–linked antibody, Cell Signaling Technology, 7074. FLAG-M2 affinity gel was obtained from Sigma-Aldrich.

Protein purifications

The Rag GTPase heterodimer was expressed and purified based on an established protocol (12). In brief, His$_6$-R$_{10}$-SU-MO-tagged RagA was coexpressed with RagC in BL21(DE3) Escherichia coli strain. The Rag GTPase dimer was sequentially passed through nickel-nitrilotriacetic acid, Mono S, Mono Q, and Superose 6 columns to obtain pure protein for biochemical analysis.

The GATOR1 complex was expressed and purified based on an established protocol (18). In brief, FLAG-tagged Depdc5 was coexpressed with HA-tagged Nprl2 and Nprl3 in FreeStyle 293-F cells. The GATOR1 complex was passed through FLAG-M2 and Superose 6 columns to obtain pure protein for biochemical analysis.

Stimulated GTP hydrolysis assay

Kinetic analysis was performed using established protocols (12, 18, 23). A single-turnover assay was carried out using 50 nM Rag GTPase heterodimer with increasing amounts of GATOR1 complex. Time points were taken to trace the reaction process (e.g. Fig. 2, B and C), and the observed rate constants were fit against GATOR1 concentration to extract $k_{cat}$ and $K_{1/2}$ values (e.g. Fig. 2D). All the experiments were repeated two to three times, and mean ± S.D. is reported.

Coimmunoprecipitation experiments

Coimmunoprecipitation experiments were performed based on an established protocol (12). In brief, two to three million
HEK-293T cells were plated onto a 10-cm Petri dish. 24 h later, the cells were transfected with cDNAs. 36 h later, cells were lysed with Triton lysis buffer (40 mM NaHEPES, pH 7.4, 5 mM MgCl₂, 10 mM Na₄P₂O₇, 10 mM sodium β-glycerol phosphate, 1% Triton X-100, and one tablet of protease inhibitor mixture/25 ml of buffer). The lysates were incubated with FLAG-M2 affinity gel and washed with Triton lysis buffer supplemented with 500 mM NaCl. Immunoprecipitated proteins were denatured by SDS buffer, resolved by SDS-PAGE, and analyzed by immunoblotting. For the amino acid stimulation experiments, HEK-293T cells were starved in RPMI 1640 medium without amino acids for an hour and restimulated with amino acids for 15 min before preparing lysates for coimmunoprecipitation analyses.

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Figure 4. The GAP mode of GATOR1 is conserved in yeast. A–C, protein sequence conservation of the GATOR1 subunits. D, stimulated GTP hydrolysis of yeast Gtr proteins by human GATOR1. WT GATOR1 and GATOR1(Depdc5(Y775A)) both stimulate GTP hydrolysis by the Gtr proteins. E, summary of kinetic parameters in D, data for the Rag GTPases were taken from Figs. 2 and 3 for comparison. F, conservation of surface-exposed residues on GATOR1. The catalytic pocket shows the highest degree of conservation.
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