Cyclic AMP Controls mTOR through Regulation of the Dynamic Interaction between Rheb and Phosphodiesterase 4D

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The mammalian target of rapamycin complex 1 (mTORC1) is a molecular hub that regulates protein synthesis in response to a number of extracellular stimuli. Cyclic AMP (cAMP) is considered to be an important second messenger that controls mTOR; however, the signaling components of this pathway have not yet been elucidated. Here, we identify cAMP phosphodiesterase 4D (PDE4D) as a binding partner of Rheb that acts as a cAMP-specific negative regulator of mTORC1. Under basal conditions, PDE4D binds Rheb in a noncatalytic manner that does not require its cAMP-hydrolyzing activity and thereby inhibits the ability of Rheb to activate mTORC1. However, elevated cAMP levels disrupt the interaction of PDE4D with Rheb and increase the interaction between Rheb and mTOR. This enhanced Rheb-mTOR interaction induces the activation of mTORC1 and cap-dependent translation, a cellular function of mTORC1. Taken together, our results suggest a novel regulatory mechanism for mTORC1 in which the cAMP-determined dynamic interaction between Rheb and PDE4D provides a key, unique regulatory event. We also propose a new role for PDE4 as a molecular transducer for cAMP signaling.

Cyclic AMP (cAMP) is a second messenger that is involved in intracellular signaling in response to a number of membrane-impermeable hormones (61, 80). cAMP plays a fundamental role in a multitude of cellular processes, including gene transcription, cell adhesion, and ion channel gating (9, 81, 90). cAMP levels are delicately regulated by the coordinated control of its rate of synthesis via adenyl cyclase activity and its rate of degradation via a large family of cAMP-hydrolyzing phosphodiesterases (PDEs) (9, 16, 31, 49). Of these PDEs, the cAMP-specific PDE4 family is widely expressed and is the current therapeutic target of selective PDE4 inhibitors for the treatment of inflammatory diseases, such as asthma and chronic obstructive pulmonary disease, as well as depression and cognitive deficits (31, 34). Four gene families encode the large family of PDE4 isoforms, which have similar catalytic activities but distinct cellular functions. These differences are due to differences in specific intracellular targeting and signaling complex formation with various binding partners, which generate the temporal and spatial dynamics of cAMP levels (19, 31, 32, 57, 89).

Members of the phosphodiesterase 4D (PDE4D) subfamily are widely expressed (17, 32, 33), and the functional roles of specific PDE4 isoforms are intimately connected with their ability to interact with specific binding partners, such as the scaffold proteins RACK1 (7, 94), myomegalin (87), β-arrestin (5, 50), AKAPs (20, 55, 56, 75, 82), DISC1 (58), Spectrin (18), and Ndel (15). It is now generally accepted that distinct PDE4 isoforms establish the compartmentalization of cAMP signaling in cells by shaping cAMP gradients around themselves and bound proteins, thereby controlling the function of cAMP effectors in these complexes (19, 31, 57, 63, 89). However, it is also accepted that PDE4 isoforms can undergo conformational changes in response to posttranslational modifications (1, 6, 28, 46), sequestration to scaffolds (94), and binding to inhibitors and substrates (32, 74, 85). Here, we uncover a novel functional role of a PDE4 isoform as a cAMP effector rather than through simply terminating cAMP signaling via cAMP hydrolysis.

mTOR interacts with Raptor to form mTOR complex 1 (mTORC1), which plays an essential role in protein synthesis in mammals in response to various signals, including insulin, nutrients, amino acids, and cellular energy status (37, 39, 67, 91). The best-characterized downstream effectors of mTORC1 are the two translational regulators S6 kinase 1 (S6K1) and 4E binding protein 1 (4E-BP1) (11, 12, 25). In response to upstream signals, mTORC1 directly phosphorylates S6K1 and 4E-BP1, which induces translation initiation (24, 30, 53). Although mTOR recognizes various environmental cues and each signal can regulate mTOR activity, the precise molecular mechanisms of how diverse signals control mTOR remain unclear. Indeed, even cAMP has been identified as an activator of...
mTORC1, although the details of the mechanism of mTORC1 regulation by cAMP are not well understood (43, 78).

Several upstream regulators of mTOR have been identified (23, 38, 65, 66, 86). Rheb, a member of the Ras-related small GTPases, is one of the best-characterized upstream activators of mTORC1 (2, 68, 76, 93). Like the other small GTPases, the activity of Rheb is regulated by guanine nucleotide binding status. Conversely, the best-characterized negative regulator of mTOR is the tuberous sclerosis complex (TSC1/TSC2), which has GTPase-activating protein (GAP) activity toward Rheb. A number of environmental signals, such as insulin, nutrients, and cellular energy status, are recognized by the TSC complex, which controls the guanine nucleotide binding status of Rheb and thereby regulates mTOR activity (10, 23, 38, 51, 84, 96). In addition, phosphatidic acid, phospholipase D, PRAS40, and Rag GTPase have been identified as mTOR regulators that respond to specific signals (21, 26, 65, 79, 86). However, there is still no clear relationship between cAMP signaling components and mTOR regulators.

In this study, we identified cAMP-specific PDE4D as a novel Rheb binding partner that serves as a sensor for cAMP signaling. This allows cAMP, through PDE4D, to release Rheb for the activation of mTORC1. This novel mechanism suggests that cAMP signals are transduced to mTORC1, and then to cap-dependent translation, through a novel pathway involving the dynamic interaction between PDE4D and Rheb.

MATERIALS AND METHODS

Antibodies and materials. Anti-Rheb (C19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-mTOR-phospho-pS6K1 (pS6K1) (Th389), -S6K1, -eEF2K1 (Thr56), -eEF2K1 (Thr202/ Tyr204), -extracellular signal-regulated kinase (anti-ERK), -pAKT (Ser473), -AKT antibodies and rapamycin were obtained from Cell Signaling (Beverly, MA). 3-[5-Cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS), E89, forskolin, cAMP, cyclic GMP (cGMP), GTPyS, GDPyS, isoproteinol, and anti-FLAG monoclonal antibodies were purchased from Sigma (St. Louis, MO). Compound C and PD98059 were purchased from Merck (Darmstadt, Germany). Anti-PDE4D polyclonal antibodies were made as previously reported (8). Anti- hemagglutinin (HA) 12CA5 antibodies were harvested from the supernatants of hybridoma cell lines (44). Protein A-Sepharose and protein G-Sepharose beads were purchased from RepliGen (Needham, MA) and Pierce (Rockford, IL), respectively. Protein A-Sepharose and protein G-Sepharose beads were kindly provided by Ariel F. Castro (Indiana University School of Medicine). Myc-mTOR, Myc-S6K, FLAG-TSC1, and FLAG-TSC2 were kindly provided by Ariel F. Castro (Indiana University School of Medicine). Myc-mTOR, Myc-S6K, FLAG-TSC1, and FLAG-TSC2 were kindly provided by Ariel F. Castro (Indiana University School of Medicine). Myc-mTOR, Myc-S6K, FLAG-TSC1, and FLAG-TSC2 were kindly provided by Ariel F. Castro (Indiana University School of Medicine). Myc-mTOR, Myc-S6K, FLAG-TSC1, and FLAG-TSC2 were kindly provided by Ariel F. Castro (Indiana University School of Medicine). Myc-mTOR, Myc-S6K, FLAG-TSC1, and FLAG-TSC2 were kindly provided by Ariel F. Castro (Indiana University School of Medicine).

Cell culture and transfection. HEK293 cells and TSC1/−/− and TSC1/−/− mouse embryo fibroblasts (MEFs) were maintained in DMEM containing 10% fetal bovine serum (FBS) (Cambrex, Walkersville, MD). HeLa and Ovar3 cells were maintained in DMEM containing 10% FBS (Gibco, Carlsbad, CA). SK-OV3 and T47D cells were maintained in RMPI medium containing 10% FBS (Cambrex, Walkersville, MD). Transfection was performed by using Lipofectamine according to the manufacturer's instructions. Cells were allowed to express the recombinant proteins or to knock down the target proteins by siRNA for 24 h after transfection and then were deprived of serum for an additional 24 h. The cells were subjected to Western blot or coimmunoprecipitation analysis.

Sample preparation and Western blot analysis. After harvesting of the HEK293 cells, total extracts were prepared by sonication in ice-cold lysis buffer (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM nicotinamide, 10 mM glycerol, 0.5 mM CHAPS, 1 mM phenylmethylsulfonyl fluoride [PMFS], 5 mM MgCl2, and protease inhibitor cocktail). The prepared cell extracts were spun at 14,000 rpm for 15 min, and the supernatant was subjected to Western blot or coimmunoprecipitation analysis.

Quantiﬁcation of cAMP. A cAMP-measuring kit was purchased from Neurones (Pohang, South Korea). The cAMP concentration in HEK293 cells was measured by using a [3H]cAMP competition assay for evaluating interactions between cAMP and with cAMP binding proteins according to the manufacturer's instructions.

Comunmunoprecipitation. The cell extract (1 mg) was incubated with 2 μg of the indicated antibodies and protein A-Sepharose beads or protein G-Sepharose beads. After 5 h of incubation at 4°C, the resulting pellets were washed four times with ice-cold lysis buffer, subjected to SDS-PAGE, and immunoblotted with the respective antibodies.

Rheb nucleotide binding assay. To analyze the GTP and GDP loading status of Rheb, a recombinant HA-Rheb was transfected into HEK293 cells and, cells were incubated in 0.5 mM MgCl2, 0.5 mM EDTA, 0.2% SDS, 5 mM DTT, 0.5 mM Gd3+, and 0.5 mM GTP at 68°C for 20 min and then resolved by thin-layer chromatography on polyethyleneimine (PEI) cellulose plates with 0.75 M KH2PO4 (pH 3.4). The amount of radioactive GTP and GDP was quantified with Multi Gauge software (Fuji).

In vitro kinase assay for mTORC1 activity. Recombinant Myc-mTOR and FLAG-Raptor were transfected into HEK293 cells and then immunoprecipitated by using an anti-FLAG antibody, as described previously (40). Purified 4EBP1 was used as a substrate for the in vitro kinase assays, and the activities were measured with an anti-phospho-4EBP1 antibody (Thr37/46). The kinase assay was performed with kinase buffer containing 25 mM HEPES (pH 7.4), 50 mM KCl, 10 mM MgCl2, 1 mg/ml bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), 1% Triton X-100, and protease inhibitors.

GTP bound to Raptor were released with 20 μl Rho elution buffer (5 mM EDTA, 0.2% SDS, 5 mM DTT, 0.5 mM GDP, and 0.5 mM GTP) at 68°C for 20 min and then resolved by thin-layer chromatography on polyethyleneimine (PEI) cellulose plates with 0.75 M KH2PO4 (pH 3.4). The amount of radioactive GTP and GDP was quantified with Multi Gauge software (Fuji).

Translation assay with HEK293 cells. Translation was assayed by luciferase reporter activity. With the pRMF reporter, luciferase activities were measured by using a [3H]cAMP competition assay for evaluating interactions between cAMP and with cAMP binding proteins according to the manufacturer's instructions.
cAMP activates mTORC1 signaling via Rheb. (A) cAMP activates mTORC1 signaling in a rapamycin-dependent manner. HEK293 cells were incubated with 10% fetal bovine serum for 36 h. After 24 h of serum deprivation, the cells were preincubated with or without serum-free medium containing 10 nM rapamycin for 45 min and were then treated with 10 μM forskolin for 5 min. The cells were lysed with buffer containing 0.5% CHAPS. Equal amounts of total cell lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of two independent experiments. (B) Forskolin activates mTORC1 in a dose-dependent manner. After 24 h of serum deprivation, HEK293 cells were treated with the indicated concentrations of forskolin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. (C) cAMP-mediated mTORC1 activation is dependent on Rheb. HEK293 cells were transfected with either the control or Rheb shRNA by using Lipofectamine. After 24 h, cells were depleted of serum for 24 h and then treated with 10 μM forskolin for 5 min. The lysates were immunoprecipitated (IP) with anti-Rheb antibodies. The immunoprecipitates and lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies.

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sured by using a dual-luciferase reporter assay system (Labsystems, Ramsey, MN). Equal amounts of extract were used to assay the cap-dependent translation of Renilla luciferase (R-Luc) or IRES-dependent translation of firefly luciferase (F-Luc). Cap-dependent translation was calculated by normalizing the R-Luc activity to the F-Luc activity as described previously (13, 47).

RESULTS

cAMP activates mTORC1 through Rheb. To examine the relationship between cAMP and mTORC1 and determine whether cAMP increases mTORC1 activity, we investigated cAMP-dependent mTORC1 activation and the requirement of Rheb in cAMP-mediated mTORC1 signaling. As shown in Fig. 1A, we confirmed (42, 78), using forskolin, which is a direct activator of adenylyl cyclase, that cAMP augmented the phosphorylation of S6K1 (T389) and 4EBP1 (T37/46), the best-characterized downstream effectors of mTORC1 (Fig. 1A). However, in these cells, cAMP did not alter the phosphorylation of Akt (S473), a downstream effector of mTORC2 (Fig. 1A). Furthermore, cAMP-mediated mTORC1 activation was reduced by treatment with rapamycin, an mTORC1 inhibitor. However, rapamycin had no effect on either Akt or Erk (T202/ Y204) phosphorylation. Despite this result, we found that mTORC1 activity was enhanced by increasing concentrations of the adenylyl cyclase activator forskolin (Fig. 1B). Indeed, mTORC1 activity was increased by cAMP-elevating agonists, such as isoproterenol and epinephrine (data not shown).

Next, we evaluated whether cAMP-mediated mTORC1 activation requires Rheb activity. Indeed, we found that the activation of mTORC1 by cAMP decreased when Rheb was silenced (Fig. 1C). Furthermore, the overexpression of Rheb-D60I, a GDP-bound dominant negative form, diminished the levels of phospho-S6K1 and phospho-4EBP1 (Fig. 1D).

We investigated whether TSC, the GAP for Rheb, has an effect on cAMP-mediated mTORC1 activation. As shown in Fig. 1E, treatment with forskolin elevated the levels of phospho-S6K1 in TSC1−/− MEFs (Fig. 1E). However, both the expression and phosphorylation levels of S6K1 were highly elevated in TSC−/− MEFs, while mTORC1 activity in TSC−/− MEFs was not affected by forskolin (Fig. 1E). Indeed, the coexpression of TSC1 and TSC2 had little effect on cAMP-mediated mTORC1 activation (Fig. 1F). These results suggest that TSC does not provide a main pathway for cAMP-mediated mTORC1 regulation.

It was reported previously that cAMP can regulate a number of signaling pathways, including AMP-activated protein kinase (AMPK) and mitogen-activated protein kinase (MAPK), which control mTORC1 (36, 77, 95). To examine the relationship between cAMP and known pathways that control mTORC1, we checked cAMP-dependent mTORC1 activation under conditions where either the AMPK or the MAPK pathways were inhibited. The overexpression of the dominant negative form of recombinant AMPKα1 increased basal phosphorylation levels of S6K1, while forskolin treatment elicited an increase in phospho-S6K1 levels similar to those seen for transfections with the vector control (Fig. 1G). Furthermore, PD98059, a MAPK inhibitor, did not affect cAMP-mediated mTORC1 regulation (Fig. 1H). These results indicate that cAMP-mediated mTORC1 activation requires other distinct mechanisms in addition to TSC, AMPK, and MAPK pathways.

Protein kinase A (PKA) is a major target of cAMP (62), and certain reports have shown that cAMP-mediated mTORC1 signaling might be affected by PKA (78). Therefore, we investigated whether cAMP-mediated mTORC1 activation required PKA in our experimental system. However, we found that H89, a PKA inhibitor, did not have a significant effect on mTORC1 activity (Fig. 1I). These results suggest that the cAMP signal that we observed is linked to mTOR through Rheb but not through either TSC or PKA.

PDE4D regulates cAMP-mediated mTORC1 activity. Next, we tested whether an elevated cellular cAMP level would affect mTORC1 activity. PDE4D provides the majority of cAMP phosphodiesterase activity (>80%) in HEK293 cells (50). siRNA reagents directed toward PDE4D can efficiently knock down the expression and, hence, the activity of all isoforms within this subfamily without affecting the expression levels of other PDE4 subfamily members in HEK cells (50). Using forskolin, we observed a sustained increase in the cellular cAMP levels of PDE4D-silenced cells (50; data not shown) and an elevation in levels of cAMP-mediated mTORC1 activity (Fig. 2A).

SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of two independent experiments. CTL, shRNA vector control. (D) The level of cAMP-mediated mTORC1 activation is decreased by a Rheb dominant negative mutant. HEK293 cells were transfected with either a GFP vector or GFP-Rheb D60I by using Lipofectamine. After 24 h, cells were deprived of serum for 24 h and then treated with 10 μM forskolin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of three independent experiments. (E) cAMP-mediated mTORC1 activation is not affected in TSC1-deficient cells. TSC1+/+ and TSC1−/− MEFs were depleted of serum for 8 h and then treated with 10 μM forskolin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of three independent experiments. (F) Overexpression of TSC1 and TSC2 has little effect on forskolin-dependent mTORC1 activation. HEK293 cells were transfected with control or recombinant TSC1/2. After 24 h, cells were deprived of serum for 24 h and then treated with 10 μM forskolin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of three independent experiments. Vec, vector control. (G) Overexpression of the AMPKα1 dominant negative form does not affect forskolin-dependent mTORC1 activation. HEK293 cells were transfected with the control or recombinant form of dominant-negative AMPKα1. After 24 h, cells were deprived of serum for 24 h and then treated with 10 μM forskolin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of two independent experiments. (I) Inhibition of PKA does not affect forskolin-dependent mTORC1 activation. After 24 h of serum deprivation, HEK293 cells were preincubated with or without serum-free medium containing PD98059 for 30 min and then treated with 10 μM forskolin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of two independent experiments.
Since the PDE4D5 isoform is the major species present in HEK293 cells (50), we analyzed whether the overexpression of recombinant forms of either wild-type (wt) PDE4D5 or a catalytically inactive PDE4D5 mutant (D556A-PDE4D5) would affect mTORC1 activity. The overexpression of wt PDE4D5 but not D556A-PDE4D5 rapidly reduced intracellular cAMP levels (Fig. 2B) and PKA activity (50, 60). However, although wt PDE4D5 overexpression decreased cAMP-mediated mTORC1 activity (Fig. 2C), unexpectedly, we found that D556A-PDE4D5 also reduced mTORC1 activation (Fig. 2C). Note that D556A-PDE4D5 has no observable PDE activity and does not decrease intracellular cyclic AMP levels in HEK293 cells (Fig. 2B) (4, 50), since Asp556 provides a critical component of the catalytic active site (32). These results suggest that PDE4D5 may have a negative role in regulating mTORC1 activation that is independent of its enzymatic activity.

**PDE4D interacts with Rheb.** Considering the observation that PDE4D5 inhibits cAMP-mediated mTORC1 activation independently of its catalytic activity, we hypothesized that PDE4D5 may have an inhibitory role in mTORC1 signaling through the association with and inhibition of a component of the mTORC1 signaling pathway rather than through reducing cAMP levels. Since Rheb is the immediate upstream mediator of mTOR signaling by cAMP, we evaluated the possibility that PDE4D5 and Rheb might interact. Ectopically expressed, recombinant PDE4D5 and Rheb coimmunoprecipitated (Fig. 3A). Furthermore, by coimmunoprecipitation, we also demonstrated that endogenous PDE4D5 could be found in a complex with endogenous Rheb in HEK293 cells (Fig. 3B) and various other cell lines (Fig. 3C). Additionally, Rheb was also able to interact with D556A-PDE4D5 (Fig. 3D). Furthermore, the PDE4D1 short form and the PDE4D2 supershort form also

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**FIG. 2.** PDE4D inhibits cAMP-mediated mTORC1 signaling. (A) PDE4D knockdown enhances mTORC1 activity. HEK293 cells were transfected with either control or PDE4D siRNA. After 24 h, cells were deprived of serum for 24 h and then treated with 10 μM forskolin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The bar graph at the bottom shows the quantification of S6K1 phosphorylation. The results shown are representative of three independent experiments. Luc, luciferase control. (B) The D556A-PDE4D5 mutant has no enzymatic activity. HEK293 cells were transfected with the indicated constructs. After 24 h, cells were deprived of serum for 24 h and then treated with 10 μM forskolin for the indicated times. The cAMP concentration in HEK293 cells was measured by using a [3H]cAMP competition assay. The results shown are representative of two independent experiments. (C) Overexpression of both wt PDE4D5 and catalytically inactive mutant (D556A) PDE4D5 inhibits mTORC1 activity. HEK293 cells were transfected with the indicated constructs and Myc-S6K1. After 24 h, cells were deprived of serum for 24 h and then treated with 10 μM forskolin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The bar graph at the bottom shows the quantification of S6K1 phosphorylation. The results shown are representative of two independent experiments.
coimmunoprecipitated with Rheb (Fig. 3E), suggesting that this binding is not an isoform-specific property but is associated with regions common to all isoforms, which differ only in their N-terminal regions.

Because Rheb is a GTPase, we investigated whether the association with and inhibition by PDE4D extended to other members of the GTPase superfamily or was binding specific for Rheb. Thus, we analyzed the interaction of PDE4D5 with several related small GTPases and the interaction of Rheb with other PDE isoforms. Although PDE4D5 immunoprecipitated with Rheb, it did not immunoprecipitate with either Ras or Rap2A (Fig. 3F), and Rheb did not interact with PDE5A (Fig. 3G). These results suggest that PDE4D interacts only with specific GTPases.

cAMP inhibits the interaction between Rheb and PDE4D. To identify the region of PDE4D responsible for interactions with Rheb, we generated glutathione S-transferase (GST) fusions of PDE4D fragments (Fig. 4A). Using purified His-Rheb and the GST-PDE4D fragments, we found that the catalytic domain of PDE4D (F2) is responsible for binding to Rheb (Fig. 4B). Because the catalytic domain of PDE4D is also the binding region for cAMP, we tested whether elevated cAMP levels affected the association and inhibition of Rheb by PDE4D5. As shown in Fig. 4C, the Rheb-PDE4D interaction was decreased upon the addition of cAMP in vitro. The catalytic activity of PDE4D is not involved in the dynamic interaction with Rheb, since the addition of cAMP similarly caused a dissociation of Rheb from GST-F2-D556A (Fig. 4C). This in-

FIG. 3. PDE4D5 specifically interacts with Rheb. (A) Interaction between recombinant PDE4D5 and Rheb. HEK293 cells were transfected with wild-type FLAG-PDE4D5 and HA-Rheb. After 36 h, the cell lysates were immunoprecipitated with either anti-HA or anti-FLAG antibodies. Both PDE4D5-bound Rheb and Rheb-bound PDE4D5 were analyzed by anti-HA immunoblotting and anti-FLAG immunoblotting, respectively. The results shown are representative of two independent experiments. (B) Confirmation of the interaction between endogenous PDE4D5 and Rheb. HEK293 cells were immunoprecipitated with Rheb-specific or control goat IgG antibodies. Coimmunoprecipitated PDE4D5 was analyzed by anti-PDE4D antibodies. The results shown are representative of two independent experiments. (C) Rheb interacts with PDE4D5 in various cell lines. Each cell lysate was immunoprecipitated with Rheb-specific or control goat IgG antibodies. Coimmunoprecipitated PDE4D5 was analyzed by anti-PDE4D antibodies. The results shown are representative of two independent experiments. (D) Interaction between the PDE4D5 catalytically inactive mutant and Rheb. HEK293 cells transfected with wt PDE4D5 or D556A-PDE4D5 were immunoprecipitated with anti-FLAG antibodies. Coimmunoprecipitated PDE4D5 was analyzed by anti-FLAG antibodies. The results shown are representative of two independent experiments. (E) Rheb interacts with all of the PDE4D splicing variants. HEK293 cells were transfected with the indicated constructs. After 36 h, the cell lysates were immunoprecipitated with anti-FLAG antibody. Coimmunoprecipitated PDE4D5 was analyzed by anti-FLAG antibodies. The results shown are representative of two independent experiments. (F) PDE4D5 interacts with Rheb but not other small GTPases. HEK293 cells were transfected with the indicated constructs. After 36 h, the cell lysates were immunoprecipitated with anti-HA antibodies. Coimmunoprecipitated PDE4D5 was analyzed by anti-FLAG antibodies. The results shown are representative of two independent experiments. (G) Rheb interacts with PDE4D5 but not PDE5A. HEK293 cells were transfected with the indicated constructs. After 36 h, the cell lysates were immunoprecipitated with anti-HA antibodies. Coimmunoprecipitated PDE4D5 and mPDE5A were analyzed by anti-FLAG antibodies. The results shown are representative of two independent experiments.
**FIG. 4.** cAMP-mediated dissociation of PDE4D from Rheb enhances the formation of the Rheb-mTOR complex. (A) Schematic diagram of the GST-PDE4D fragments. (B) Rheb interacts directly with the catalytic domain of PDE4D. Each of the GST-PDE4D fragments was incubated with purified His-Rheb. After performing the GST pulldown assay, bound Rheb was analyzed by anti-Rheb antibodies, and the amounts of the GST-PDE4D fragments are shown by Ponceau S staining. The results shown are representative of two independent experiments. (C) cAMP, a substrate of PDE4D, specifically disrupts the interaction between Rheb and PDE4D. GST, GST-F2 wt, or D556A mutant fragments were incubated with His-Rheb in the presence of the indicated concentrations of cAMP or cGMP. Bound Rheb was analyzed by anti-Rheb antibodies, and the amounts of GST-F2 are shown by Ponceau S staining. The results shown are representative of two independent experiments. (D) PDE4D dissociates from Rheb with increased cellular cAMP levels. HEK293 cells were treated with 10 μM forskolin for the indicated times. The cell lysates were immunoprecipitated with anti-Rheb antibodies. The results shown are representative of two independent experiments. (E) PKA has no effect on the dynamic interaction between PDE4D and Rheb. After 24 h of serum deprivation, HEK293 cells were preincubated with or without serum-free medium containing 30 μM H89 for 45 min and then treated with 10 μM forskolin for 5 min. The cell lysates were immunoprecipitated with anti-Rheb antibodies. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. (F) cAMP specifically decreases the interaction between Rheb and PDE4D. HEK293 cells were treated with 10 nM insulin, 10 μM isoproterenol, or 10 μM forskolin for 5 min. The cell lysates were immunoprecipitated with anti-Rheb antibodies. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of two independent experiments.
interaction was not affected by the addition of cGMP (Fig. 4C), which neither is hydrolyzed by nor binds to PDE4 (32).

To confirm the dynamic interaction, we tested whether cAMP regulates the interaction between PDE4D and Rheb in a cellular system. The interaction of endogenous PDE4D with Rheb was strongly reduced under conditions of elevated cAMP cellular levels, which was achieved by treatment with the adenylyl cyclase activator forskolin (Fig. 4D). The PKA inhibitor H89 had no effect on the ability of the elevation of cAMP levels to disrupt the interaction between PDE4D5 and Rheb, which correlated with the mTORC1 activity (Fig. 4E). We also observed a similar result when isoproterenol, a β-adrenergic receptor agonist that increases cAMP levels, was used to challenge these cells (Fig. 4F). The interaction was not affected by insulin treatment (Fig. 4F).

Next, we analyzed whether the PDE4D5-Rheb interaction was regulated by the guanyl-nucleotide status of Rheb. However, this interaction did not depend on the GTP/GDP status of Rheb (Fig. 5A). Furthermore, forskolin treatment and the overexpression of PDE4D5 had no effect on the GTP/GDP ratio of Rheb (Fig. 5B and C). These results suggest that cAMP, rather than the guanyl-nucleotide switching of Rheb,

FIG. 5. PDE4D5 does not affect the guanine nucleotide-bound status of Rheb. (A) The interaction between Rheb and PDE4D does not depend on its nucleotide binding status. His-Rheb was charged with 100 mM GDP or 100 mM GTP. GST or GST-F2 fragments were incubated with free or nucleotide-charged His-Rheb. After performing the GST pulldown assay, bound Rheb was analyzed by an anti-Rheb antibody, and the amounts of GST-F2 are shown by Ponceau S staining. (B) cAMP does not increase the GTP/GDP ratio of Rheb. HEK293 cells were transfected with HA-Rheb. After 24 h, cells were deprived of serum for 20 h and incubated with 0.5 mCi 32P for 4 h and then treated with 10 μM forskolin for 5 min. The lysates were subjected to an in vivo radiolabeling assay. The results shown are representative of two independent experiments. (C) Overexpression of PDE4D5 does not affect the GTP/GDP ratio of Rheb. HEK293 cells were transfected with the indicated constructs. After 36 h, cells were incubated with 0.5 mCi 32P, for 4 h and subjected to an in vivo radiolabeling assay. The results shown are representative of two independent experiments.
may be a direct regulator of the dynamic interaction between PDE4D and Rheb.

**PDE4D negatively regulates mTORC1.** Interestingly, forskolin treatment enhanced the interaction between Rheb and mTOR. HEK293 cells were treated with 10 μM forskolin or 10 μM isoproterenol for 5 min. The cell lysates were immunoprecipitated with anti-Rheb antibodies. The immunoprecipitates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of two independent experiments. (B) The PDE4D F2 fragment inhibits mTORC1 kinase activity. Myc-mTOR and FLAG-Raptor were transfected into HEK293 cells. After immunoprecipitation with anti-FLAG antibodies, immunoprecipitates and GTPγS-loaded GST-Rheb were incubated in the absence or presence of the F2 fragment at 37°C for 10 min. Proteins were resolved by SDS-PAGE, and the phosphorylation of 4EBP1 on Thr37/46 was analyzed by Western blotting. The bar graph at the bottom shows the quantification of 4EBP1 phosphorylation. The results shown are representative of three independent experiments. (C) mTORC1 activity inhibited by the F2 fragment is restored by cAMP but not cGMP. Myc-mTOR and FLAG-Raptor were transfected into HEK293 cells. After immunoprecipitation with anti-FLAG antibodies, immunoprecipitates, GTPγS-loaded GST-Rheb, and the F2 fragment were incubated in the absence or presence of cAMP or cGMP at 37°C for 10 min. Proteins were resolved by SDS-PAGE, and the phosphorylation of 4EBP1 on Thr37/46 was analyzed by Western blotting. The bar graph at the bottom shows the quantification of 4EBP1 phosphorylation. The results shown are representative of two independent experiments. (D) The PDE4D F2 fragment inhibits mTORC1 activity. HEK293 cells were transfected with the indicated constructs. After 36 h, the cells were lysed, subjected to SDS-PAGE, and immunoblotted with the respective antibodies. The bar graph at the bottom shows the quantification of S6K1 phosphorylation. The results shown are representative of three independent experiments. (E) The PDE4D F2 fragment inhibits mTORC1 activity. HEK293 cells were transfected with the indicated constructs and Myc-S6K1. After 24 h, cells were deprived of serum for 24 h and then treated with 10 μM forskolin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The bar graph at the bottom shows the quantification of S6K1 phosphorylation. The results shown are representative of two independent experiments. (F) PDE4D knockdown does not affect insulin-mediated mTORC1 activity. HEK293 cells were transfected with either control or PDE4D siRNA. After 24 h, cells were deprived of serum for 24 h and then treated with 20 nM insulin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. (G) PDE4D wt overexpression does not affect insulin-mediated mTORC1 activity. HEK293 cells were transfected with either the control or wt PDE4D constructs. After 24 h, cells were deprived of serum for 24 h and then treated with 20 nM insulin for 5 min. The lysates were subjected to SDS-PAGE and immunoblotted with the respective antibodies. (H) PDE5A does not affect mTORC1 activity. HEK 293 cells were transfected with the indicated constructs. After 36 h, the cells were lysed, subjected to SDS-PAGE, and immunoblotted with the respective antibodies. 4D5, PDE4D5; m5A, mPDE5A.
major cellular downstream target of mTORC1 is the regulation of cap-dependent translation, in which the phosphorylation of 4EBP1 by mTORC1 releases 4EBP1 from eIF-4E for translation initiation (52). To further confirm the role of PDE4D in mTORC1 signaling, we evaluated cap-dependent translation by measuring the effect of cAMP on 4EBP1-cap binding. 4EBP1 was dissociated from eIF-4E upon the treatment of cells with either forskolin or insulin (Fig. 7A). Pre-treatment with rapamycin did not induce a cAMP-mediated dissociation of 4EBP1 from eIF-4E. We found that the silencing of PDE4D decreased the interaction of 4EBP1 with eIF-4E, whereas the overexpression of either wt PDE4D or the PDE4D catalytic region (F2) increased the interaction of 4EBP1 with eIF-4E, whereas the overexpression of either wt PDE4D or the PDE4D catalytic region (F2) increased the interaction of 4EBP1 with eIF-4E (Fig. 7B and D). This result correlated with the regulation of mTORC1 activity by PDE4D5 (Fig. 2C and 6D). We also found that PDE4D had a negative effect on cap-dependent translation, as measured by the relative luciferase assay (Fig. 7C and E). These results support the hypothesis that PDE4D is a cAMP-dependent regulator not only of mTORC1 activation but also of cap-dependent translation, a pivotal cellular downstream process regulated by mTORC1.

DISCUSSION

Here, we made the novel discovery that cAMP signaling is linked to the mTORC1 pathway via a direct interaction between PDE4D and Rheb. We propose three key aspects by which cAMP mediates mTORC1 activation. First, the cAMP signal activates mTORC1 through Rheb, rather than through either TSC or PKA, thereby inducing the initiation of cap-dependent translation. Second, we propose that the decisive mechanism of mTORC1 activity controlled by cAMP is dynamic and involves the cAMP-dependent release of Rheb from PDE4D to allow the association of Rheb with mTORC1. Third, we suggest that PDE4D serves as a molecular sensor that mediates the cAMP regulation of mTORC1. In conclusion, our findings suggest that the dynamic PDE4D-Rheb interaction is a key mediator of cAMP-mediated mTORC1 activation.

These findings have led us to suggest a novel mechanism for Rheb in cAMP-induced mTORC1 activation. Since Rheb is the immediate upstream activator of mTOR and is regulated by various signals via the GAP activity of TSC, it has been considered to provide a molecular gate that transmits signals to mTOR (10, 23, 38, 48, 51, 68, 76, 84, 96). Although most investigations have focused on the negative control of Rheb by the GAP activity of TSC, several investigators proposed that Rheb regulation is independent of TSC (3, 35, 44, 45). Indeed, a recent study suggested that translationally controlled tumor protein (TCTP) functions as a guanine nucleotide exchange factor (GEF) for Rheb, which results in the activation of mTORC1 (35). Another study demonstrated that Bnip3, a hypoxia-inducible Bcl-2 homology 3 domain-containing protein, mediates the hypoxia-induced inhibition of mTOR by interacting with Rheb (45). Additionally, FKBPs (immunophillin FK506 binding protein, 38kDa) has been identified as a direct binding partner of Rheb and an inhibitor of mTORC1 (3), and in our previous work (44), we showed that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) could regulate mTORC1 activity through direct interactions with Rheb in response to glycolytic flux. The study that we describe here suggests that the novel cAMP-mediated regulatory mechanism of Rheb is independent of both the GAP activity of TSC and the guanine nucleotide binding status of Rheb (Fig. 1E and 5A to C). Thus, Rheb may be a central mediator that links diverse signals to the mTORC1 pathway.

cAMP-specific PDEs are known to negatively regulate cAMP signaling via the hydrolysis of cAMP (16, 31). Of the various PDE families that hydrolyze cAMP, members of the PDE4 family are ubiquitously expressed, play a key role in maintaining the compartmentalization of cAMP signaling, and, from the use of selective inhibitors and knockdown studies, have been shown to play key roles in a variety of important processes such as inflammation, learning, and memory (16, 17, 31–33). Although all PDE4 family members have similar structures in the catalytic domains and similar enzymatic activities, each PDE4 isoform is characterized by a unique N-terminal region that invariably facilitates targeting to specific protein complexes (31–33). Complex formation with particular binding partners enables PDE4s to target particular intracellular locations or particular cytosolic signaling complexes, which regulate localized intracellular cAMP levels, modulate the susceptibility of sequestered cAMP effectors to elevations in cAMP levels, and confer functional specificity (31, 33). Here, we show that PDE4D, and in particular PDE4D5, can interact with Rheb and thereby serve as a cAMP-specific negative regulator of mTORC1, independent of its enzymatic activity. In agreement with this novel paradigm for cAMP signaling, we have shown that GAPDH regulates mTORC1 activity through direct interaction with Rheb in response to glycolytic flux (44), and indeed, PDE7A1 has been shown to inhibit PKA through interactions with the PKA C domain independently of its cAMP hydrolysis activity (27).

When the cellular cAMP level rises, its two central effectors are PKA (81, 83) and exchange protein activated by cAMP (EPAC) (9). PKA phosphorylates the long PDE4D isoforms, thereby activating the short-term hydrolysis activity of PDE4D and contributing a major part of the cellular desensitization process to cAMP (54, 59, 70, 71, 89). However, PKA is clearly not involved in the novel function of PDE4D5 that we report here because the inhibition of PKA did not affect the ability of elevated cAMP levels to disrupt the Rheb-PDE4D5 interaction, and critically, in vitro, the interaction between purified PDE4D and purified Rheb was disrupted solely by the addition of cAMP. The latter experiment unequivocally shows that the addition of cAMP to a mixture of PDE4D and Rheb severely compromises the interaction. This led us to consider that the catalytic domain of PDE4D, which binds cAMP, may itself function as a molecular sensor for changes in the cAMP level and transmit them to the mTOR signaling system. Indeed, we were able to demonstrate here that cAMP induces the dissociation of PDE4D5 from Rheb. Certainly, this would provide sensor functioning at physiological levels of cAMP, as PDE4 family members exhibit Km values for cAMP in the 1 to 4.0 μM range, which correlates with the typical ranges (1 to 30 μM) of intracellular cAMP concentrations (32). Additionally, there is a precedent for the PDE4 catalytic subunit undergoing conformational changes upon binding to various selective inhibitors (32, 34, 73, 85); it is possible that conformational changes occur upon cAMP binding to PDE4 isoforms in complexes with spe-
FIG. 7. PDE4D has a negative effect on cap-dependent translation efficiency. (A) cAMP decreases the interaction between 4EBP1 and the cap structure. After 24 h of serum deprivation, HEK293 cells were preincubated with or without serum-free medium containing 10 nM rapamycin for 45 min and were then treated with 10 μM forskolin or 20 nM insulin for 5 min. The cell lysates were incubated with 10 μl of m7-GTP Sepharose beads for 4 h. The resulting beads were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of two independent experiments. (B) PDE4D knockdown disrupts the interaction between 4EBP1 and the cap structure. HEK293 cells were transfected with either control or PDE4D siRNA. After 24 h, cells were deprived of serum for 24 h and then treated with 10 μM forskolin for 5 min. After the pulldown assay was performed, the resulting beads were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of two independent experiments. (C) PDE4D knockdown enhances cap-dependent translation. HEK293 cells were transfected with either control or PDE4D siRNA. After 24 h, cells were deprived of serum for 24 h and then treated with 10 μM forskolin for 36 h. The resulting lysates were measured by the dual-luciferase reporter assay. The results shown are representative of two independent experiments. NT, no treatment. (D) PDE4D5 overexpression enhances the interaction between 4EBP1 and the cap structure. HEK293 cells were transfected with the indicated constructs. After 36 h, the cell lysates were incubated with 10 μl of m7-GTP Sepharose beads for 4 h. The resulting beads were subjected to SDS-PAGE and immunoblotted with the respective antibodies. The results shown are representative of two independent experiments. (E) PDE4D5 overexpression inhibits cap-dependent translation. HEK293 cells were transfected with the indicated constructs. After 36 h, the resulting lysates were measured by the dual-luciferase reporter assay. The results shown are representative of two independent experiments.
sic partner proteins. All of the PDE4 crystal structures reported to date have focused on the core catalytic unit with various bound inhibitors (88). However, there are no crystal structures of either full-length PDE4 or the enzyme in complex with proteins that sequester it, and thus, we have no knowledge of structural changes from such approaches. However, binding studies of inhibitors, phosphorylation states, and the binding of partner proteins have all identified various marked changes in activity, thermostability, and inhibitor binding that are consistent with profound conformational changes (32, 33, 73). Indeed, there is a large amount of literature indicating that PDE4 isoforms can adopt multiple conformational states (32, 34, 73). We suggest here that the release of Rheb from PDE4D might thus be dynamically controlled in cells by cAMP binding. Moreover, we also observed that isoproterenol, a β-adrenergic receptor agonist that increases cAMP levels, induced the dissociation of PDE4D5 from Rheb in cells. These results imply that the regulatory mechanism of Rheb by PDE4D5 may consist of the binding of cAMP to the PDE4D catalytic site, which then displaces Rheb from PDE4D5. Whether this occurs though competition between Rheb and cAMP for binding to PDE4D5 or through an allosteric conformational change remains to be determined. It is interesting that the RACK1 signaling scaffold binds to PDE4D5 not only at a site within its unique N-terminal domain but also at the catalytic domain, where it abuts the cAMP binding site (7) and causes a conformational change that is detectable through an altered binding of the inhibitor rolipram to this enzyme (94). A crystal structure of the complex of PDE4D and Rheb may provide critical additional details of the cross talk between the cAMP and mTOR pathways.

mTORC1 is a molecular hub that receives various signals from the extracellular environment to modulate protein synthesis. Cells may have developed multiple ways to regulate mTORC1 signaling in response to diverse environmental cues. The best-characterized upstream regulators of mTORC1 are TSC and Rheb. In addition to regulation by TSC-Rheb, PRA540 and Rag GTPase have been identified as mTORC1 regulators in response to insulin and amino acids, respectively (65, 66, 86). Here, we have identified a novel function for PDE4D in cAMP-mediated mTORC1 activation. According to our model, PDE4D has dual roles as a specific sensor of cAMP and a cAMP-hydrolyzing enzyme. When the cellular cAMP level rises, PDE4D recognizes the increased level and dissociates from Rheb, thereby enhancing the interaction between Rheb and mTOR. Simultaneously, free PDE4D may function as a negative-feedback regulator via the hydrolysis of cAMP that surrounds mTORC1. Thus, cells may have an advanced mechanism that allows mTORC1 to immediately respond to the cAMP signal via the dual function of PDE4D. The possible negative regulation of PDE4D by Rheb binding under basal conditions and of the cAMP-induced activation of PDE4D may be interesting for future studies aimed at elucidating additional details of the cross talk between the cAMP and mTOR pathways.

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