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Gβγ interacts with mTOR and promotes its activation

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ABSTRACT

Diverse G protein-coupled receptors depend on Gβγ heterodimers to promote cell polarization and survival via direct activation of PI3Kγ and potentially other effectors. These events involve full activation of AKT via its phosphorylation at Ser473, suggesting that mTORC2, the kinase that phosphorylates AKT at Ser473, is activated downstream of Gβγ. Thus, we tested the hypothesis that Gβγ directly contributes to mTOR signaling. Here, we demonstrate that endogenous mTOR interacts with Gβγ. Cell stimulation with serum modulates Gβγ interaction with mTOR. The carboxyl terminal region of mTOR, expressed as a GST-fusion protein, including the serine/threonine kinase domain, binds Gβγ heterodimers containing different Gβ subunits, except $G\beta_4$. Both, mTORC1 and mTORC2 complexes interact with $G\beta_1\gamma_2$ which promotes phosphorylation of their respective substrates, p70S6K and AKT. In addition, chronic treatment with rapamycin, a condition known to interfere with assembly of mTORC2, reduces the interaction between Gβγ and mTOR and the phosphorylation of AKT; whereas overexpression of Gαi interfered with the effect of Gβγ as promoter of p70S6K and AKT phosphorylation. Altogether, our results suggest that Gβγ positively regulates mTOR signaling via direct interactions and provide further support to emerging strategies based on the therapeutical potential of inhibiting different Gβγ signaling interfaces.

1. Introduction

G-protein coupled receptors (GPCRs) constitute the most abundant group of plasma membrane receptors. They control multiple fundamental biological processes including neurotransmission, metabolism, blood pressure, vascular permeability and chemotaxis, among others, that are therapeutically targeted with ligands that affect GPCR activation and signaling. The existence of many orphan GPCRs and the discovery of new G protein-dependent and independent effectors maintain their pharmacological potential far from being reached [1,2]. A common feature of GPCR signaling is the participation of heterotrimeric G proteins that activate, via direct interactions, protein and lipid kinases, guanine nucleotide exchange factors for Rho GTPases (RhoGEFs), phospholipases, ion channels and adenylyl cyclases, providing specificity to the numerous molecular events regulated by GPCR signaling. Heterotrimeric G proteins, constituted by a GDP-loaded Gα subunit that interacts with a Gβγ heterodimer, are the central transducers of G protein coupled receptors. Upon receptor activation, Gα exchanges GDP for GTP, dissociates from Gβγ and acquires, as well as Gβγ does, the ability to interact with their respective effectors [3,4].

A prominent role for Gβγ as a phylogenetically conserved GPCR signal transducer is well established. Thus, the potential pharmacological action of small molecules and peptides able to differentially inhibit specific Gβγ-dependent effectors is current subject of intense investigation [5,6]. For instance, Gβγ-regulated cell polarity and chemotactic migration is exacerbated in inflammation and metastasis [7–10]; whereas inhibition of Gβγ signaling prevents SDF-1α induced breast tumor cell migration and invasion [8]. In addition, inhibition of Gβγ ability to recruit PI3Kγ to the plasma membrane interferes with sphingosine-1-phosphate-dependent cell migration and angiogenesis [11]. Among the Gβγ-controlled effectors that link GPCR signaling to cytoskeleton remodeling, different components of the PI3K/AKT/mTOR/P-Rex1 signaling pathway are considered crucial. Gβγ activates lipid kinases such as PI3Kγ and PI3Kβ [12], and Rac guanine nucleotide exchange factors such as P-REX1 which, besides being a direct effector of Gβγ, is also regulated by phosphatidylinositol-3,4,5-trisphosphate [13] and

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responds to growth factors [14]. Additional effectors of Gβγ that play a relevant role in cell migration include serine/threonine and tyrosine kinases such as GRK-2 and Btk [15,16].

The serine/threonine kinase mTOR (mammalian target of rapamycin/mechanistic TOR) is a central controller of cell growth, proliferation, survival and migration [17,18]. This high molecular weight multidomain kinase constitutes the catalytic core of mTORC1 and mTORC2, two multiprotein complexes with different specificities and mechanisms of action. The basic mTORC1 complex, the paradigmatic sensor of nutrients and energy status, is allosterically inhibited by rapamycin. This complex is defined by the presence of Raptor, a dynamic interactor that keeps mTOR activity down under basal conditions and contributes to recruit its substrates upon stimulation. The rapamycin-resistant complex, named mTORC2, is characterized by the presence of Rictor; whereas both complexes contain mLST8 [19,20]. Additional mTOR-interacting proteins include PRAS40 [21], Deptor [22] and GRp58 [23], among others [24]. A mechanism by which growth factors, nutrients and energy levels control mTORC1 signaling has been described in detail [25]. However, the regulation of mTORC2, an important regulator of actin reorganization and AKT activation, is less characterized. We recently demonstrated that mTORC2 leads to Rac activation and cell migration via direct interactions with P-Rex1 [26]. In addition, Bae and colleagues found that P-Rex1 controls the ability of mTORC2 to phosphorylate AKT1, regulating invasive cancer cell migration [27,28]. Since different signaling pathways lead to AKT phosphorylation and chemotaxis, putatively via mTORC2, the existence of pathway-specific mechanisms that regulate this complex is predicted. Basic Gβγ-dependent chemotactic mechanisms occurring independently of PI3K have been described in *Dictyostelium*, suggesting the existence of downstream effectors still sensitive to the control of Gβγ [29]. Since this heterodimer directly regulates effectors linked to AKT activation and cell migration, including P-Rex1, and binds the catalytic core of several serine/threonine kinases, we explored the hypothesis that Gβγ directly interacts with mTOR, a regulator of P-Rex1 relevant in chemotactic cell migration, contributing to activate this multifunctional serine/threonine kinase in response to GPCR agonists.

2. Materials and methods

2.1. Yeast two hybrid screening

The carboxyl terminal region of mTOR, originally obtained as a prey in the yeast two hybrid system using P-Rex1-DEP-DEP domains as bait [26] was used to assess its potential interaction with $G\beta_1$, cloned as bait in the Matchmaker system III (Clontech) following the protocol described in detail elsewhere [30]. Transformants were selected in medium lacking Leu/Trp and tested for interactions in medium lacking His/Leu/Trp or lacking Ade/His/Leu/Trp and checked for α-galactosidase expression by α-X-Gal (where X-Gal is 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) assay. The specificity of the interaction was determined using pGBKT7-p53 and pGB3 empty vector as a control.

2.2. DNA constructs

pEF-His6-$G\beta_1$, pCEFL-3xFlag-$G\beta_1$, pCEFL-Flag-$G\beta_1$, pCEFL-Flag-$G\beta_2$, pCEFL-Flag-$G\beta_3$, pCEFL-Flag-$G\beta_4$, pCEFL-Flag-$G\beta_5$, pCEFL-$G\gamma_2$ and pCEFL-HA-Gα were reported previously [11]. cDNAs coding Rictor and Raptor were obtained from Addgene (plasmids 1860 and 1859). For pull down experiments, the carboxyl terminal region of mTOR, including the full kinase domain, was subcloned in frame with the sequence coding for glutathione S-transferase (GST) into the mammalian expression vector pCEFL-GST.

2.3. Cell culture and transfection

The human embryonic Kidney 293 (HEK293) and HEK293T cell lines were routinely cultured in DMEM medium (Sigma–Aldrich), supplemented with 10% fetal bovine serum and 1% glutamine, penicillin and streptomycin, in a 5% CO_2 atmosphere at 37 °C. Cells were transfected with lipofectamine Plus reagent (Life Technologies) using four micrograms of plasmid DNA per plate of cells grown to 60–70% confluence on 10-cm-diameter poly-D-lysine-coated dishes. Cells were used at 48 h post transfection.

2.4. Affinity precipitation assays

The interaction between Flag-tagged-$G\beta_1\gamma_2$ or different combinations of Flag-Gβ and Gγ with the carboxyl terminal region of mTOR, including the kinase domain, fused to GST was assessed by pull down using glutathione-Sepharose beads. Interacting Gβγ was revealed by western blotting using anti-Flag antibodies. Interaction between endogenous mTOR and His-Gβγ was assessed by Talon affinity pulldown in total cell lysates from HEK293T cells transfected with His-$G\beta_1$ and His-$G\gamma_2$. The effect of serum on this interaction was assessed using 10% FBS to stimulate HEK293T cells that were deprived of serum during 12–16 h. The effect of rapamycin on the interaction was assessed in cells incubated for 1 or 24 h with this agent. Total cell lysates were obtained with 1 ml of ice-cold buffer (50 mM Tris, pH 7.5, 0.15 M NaCl, 1%Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF) and incubated with TALON resin (Clontech) in a rocking platform for 30 min a 4 °C. The resin was collected by centrifugation and washed four times with ice-cold lysis buffer containing 5 mM Imidazole. Bound proteins were eluted by boiling for 5 min in Laemmli sample buffer and fractionated on a 6% and 10% SDS–PAGE and detected by Western blotting using anti-histidines (Sigma–Aldrich) and anti mTOR antibodies (Cell Signaling Technology).

2.5. Immunoprecipitation assays

The interaction between endogenous mTOR and Gβγ was assessed in non-transfected HEK293T cells. The potential association of Gβγ and mTORC1 and mTORC2 complexes was assessed by immunoprecipitation of transfected myc-Raptor or myc-Rictor, respectively. Cells lysates were obtained with lysis buffer and incubated for 12 h at 4 °C with 10 μg/ml anti-mTOR antibody (Santa Cruz Biotechnology). The immune complexes were recovered by incubation for 2 h at 4 °C with protein A/G plus agarose. Beads were washed four times with ice cold lysis buffer and boiled in sample buffer. Immunoprecipitated proteins were fractionated on a 6% and 10% SDS–PAGE and detected by western blotting using anti Gβ (Santa Cruz Biotechnology) and anti mTOR antibodies (Cell Signaling Technology).

2.6. Western blot assays

To test whether Gβγ played a role in the phosphorylation of mTORC1 and mTORC2 downstream substrates, HEK293 cells were transfected with His-$G\beta_1\gamma_2$ or Gα. Two days after transfection cells were serum starved for 8 h, controls included cells transfected with empty plasmids and left unstimulated or stimulated with 10 μM LPA (5 min) prior to cell lysis. The lysates were analyzed by western blotting with anti-Gβ to confirm its overexpression. Phosphorylation of mTORC2 or mTORC1 substrates, or the phosphorylation of ERK, as well as the expression of the respective proteins was

detected using the following antibodies: phospho-Akt-Ser473 and ERK from Santa Cruz; Akt form Sigma; phospho-p70^{S6K}-Thr389, p70^{S6K}, phospho-S6-Ser240/244, S6, phospho-ERK, from Cell Signaling. Peroxidase-labeled secondary antibodies were from KPL. Bands were visualized using a West Pico system (Pierce Biotechnology) or Immobilon Western chemiluminescent substrate (Millipore).

2.7. Statistical analysis

Statistical significance of the differences among data was determined by analysis of variance and Student–Newman–Keuls test using Graphpad Prism Version 2.0 software (GraphPad Software, San Diego, CA). $p < 0.05$ was considered a statistically significant difference.

3. Results

3.1. mTOR interacts with Gβγ in yeast and mammalian cells

Gβγ is a critical signal transducer that directly activates the PI3K/AKT/mTOR/P-Rex1 signaling pathway in response to chemotactic GPCRs (Fig. 1A). Here, we hypothesized that Gβγ directly interacts with mTOR contributing to its activation. We initially tested a possible direct interaction between mTOR carboxyl terminus, including the kinase domain, and Gβ in the yeast two hybrid system, which is based on the reconstitution of the GAL4 transcription factor. As shown in Fig. 1B, right panel, mTOR carboxyl terminus interacted with Gβ; this interaction was strong and specific as indicated by the growth, in high stringency restrictive media lacking adenine, histidine, leucine and tryptophan (-AHLT), of yeast

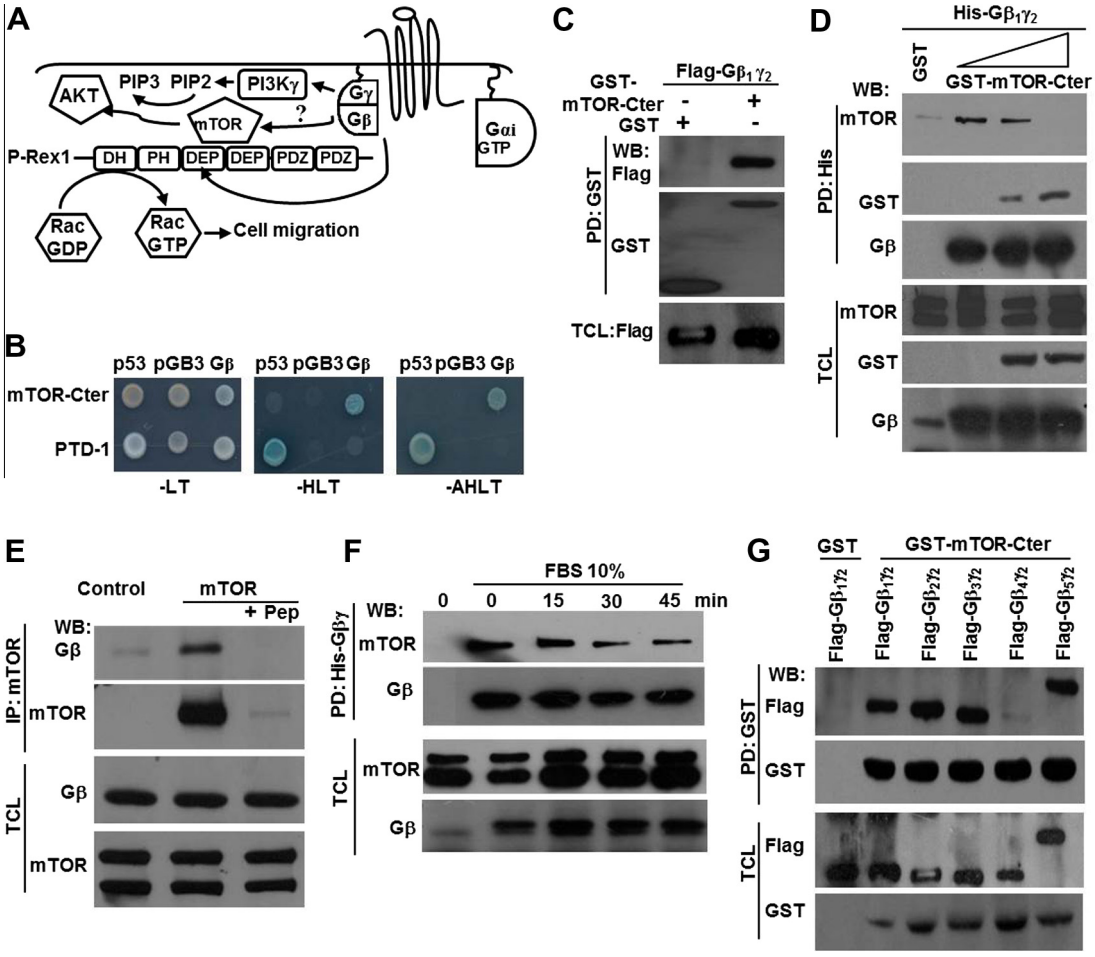

Fig. 1. Gβγ interacts with mTOR in yeast and mammalian cells. (A) Schematic representation of the Gβγ/PI3K/AKT/mTOR/P-Rex1 signaling complex indicating a possible interaction between Gβγ and mTOR. (B) Gβ interacts with mTOR in the yeast two hybrid system. The specificity of the interaction between Gβ and mTOR carboxyl terminus was determined in yeast using p53 or pGB3 as negative controls. All yeast grew in media lacking Leucine and Tryptophan (-LT) which selects for the presence of the plasmids (left panel), but only those displaying strong interaction grew under high stringency conditions (media lacking Adenine, Histidine Leucine and Tryptophan, -AHLT). (C) Gβγ interacts with mTOR carboxyl terminus in HEK293Tcells. Gβγ (tagged with 3 × Flag) and mTOR carboxyl terminus expressed as GST-fusion protein (GST-mTOR-Cter) were transfected in HEK-293T cells. GST was affinity-purified with glutathione beads, and both total cell lysates (TCL; bottom panel) and pulldowns (PD; upper panel) were resolved on SDS–polyacrylamide gels and analyzed by immunoblotting using the indicated antibodies. (D) The interaction between Gβγ and endogenous mTOR is competed with mTOR carboxyl terminus fused to GST. HEK-293T cells expressing His-Gβγ and increasing concentrations of GST-mTOR prey were used for pull down (PD) experiments. The expression of the transfected proteins and endogenous proteins was verified by western blot in total cell lysates (TCL; bottom panel) and pull downs. (E) Endogenous Gβγ interacts with endogenous mTOR. Total cell lysates from HEK-293T were used to immunoprecipitate mTOR and interacting Gβγ was revealed by western blot using anti-Gβ antibodies. The specificy of the assay was determined by using a specific peptide competing with mTOR antibodies (+Pep) or a goat antibody as negative control. (F) Effect of FBS on the interaction between mTOR and Gβγ. HEK-293T cells expressing His-Gβγ were serum starved during 16 h and then stimulated for 5, 15, 30 and 45 min with 10% FBS. His-Gβγ was purified by pull down (PD) and the presence of mTOR was detected by Western blotting. (G) Different Gβγ heterodimers, except $G\beta_4\gamma_2$, interact with mTOR carboxyl terminus. The indicated Flag-tagged Gβ subunits were cotransfected with $G\gamma_2$ and GST-mTOR-Cter in HEK-293T cells and their potential interaction was detected by GST-pull down (PD) assays followed by western blot using the indicated antibodies.

transformed with the corresponding plasmids and in media also lacking histidine (-HLT) to test for weak interactions (Fig. 1B, middle panel). Similar results were obtained with yeast expressing p53 and pTD1, used as positive controls. In parallel, yeast transformed with all the different plasmids grew in media lacking leucine and tryptophan (-LT), which selects just for the presence of the plasmids independently of possible interactions between encoded proteins (Fig. 1B, left panel). In mammalian cells, we confirmed the interaction between Gβγ and mTOR carboxyl terminus, as indicated by the presence of 3xFlag-tagged $G\beta_1\gamma_2$ in the GST-mTOR carboxyl terminus pulldown, but not in the negative control pulldown, done with lysates from HEK293T cells transfected with GST (Fig. 1C). Furthermore, full length endogenous mTOR interacted with His6-Gβγ expressed in HEK293T cells (Fig. 1D) whereas this interaction was competed with increasing doses of GST-tagged-mTOR carboxyl terminus, but not GST (Fig. 1D). Furthermore, endogenous mTOR interacted with endogenous Gβγ as revealed by immunoprecipitating mTOR and detecting Gβ. The specificity of this assay was demonstrated by preincubating mTOR antibodies with a specific blocking peptide; in this case, Gβ was not found in the resulting immunoprecipitates (Fig. 1E). To investigate whether the interaction between mTOR and Gβγ can be influenced by stimulus, we determined the interaction of His-tagged Gβγ with endogenous mTOR by pull down assays of lysates obtained from HEK293T cells initially deprived of serum for 16 h, then stimulated with 10% fetal bovine serum (FBS). The interaction between mTOR and Gβγ decreased in response to FBS (Fig. 1F).

Gβγ is a heterodimer assembled from a repertoire of 5 different Gβ-subunits and 12 Gγ-subunits, forming a variety of dimers [2]. We investigated whether Gβγ heterodimers containing different Gβ subunits could interact differentially with mTOR. As shown in Fig. 1G, all Gβγ heterodimers, except $G\beta_4\gamma_2$, interacted with GST-mTOR carboxyl terminus. Collectively, these results indicate that interaction between mTOR and Gβγ, discovered by yeast two-hybrid screening and detected by pull down of transfected epitope-tagged proteins, occurs between endogenous mTOR and Gβγ heterodimers.

3.2. Gβγ interacts with mTORC1 and mTORC2

The serine/threonine kinase mTOR constitutes the catalytic core of two independent multiprotein complexes, mTORC1 and mTORC2, identifiable by Raptor or Rictor as distinctive associated proteins, respectively [24]. In order to address whether Gβγ could

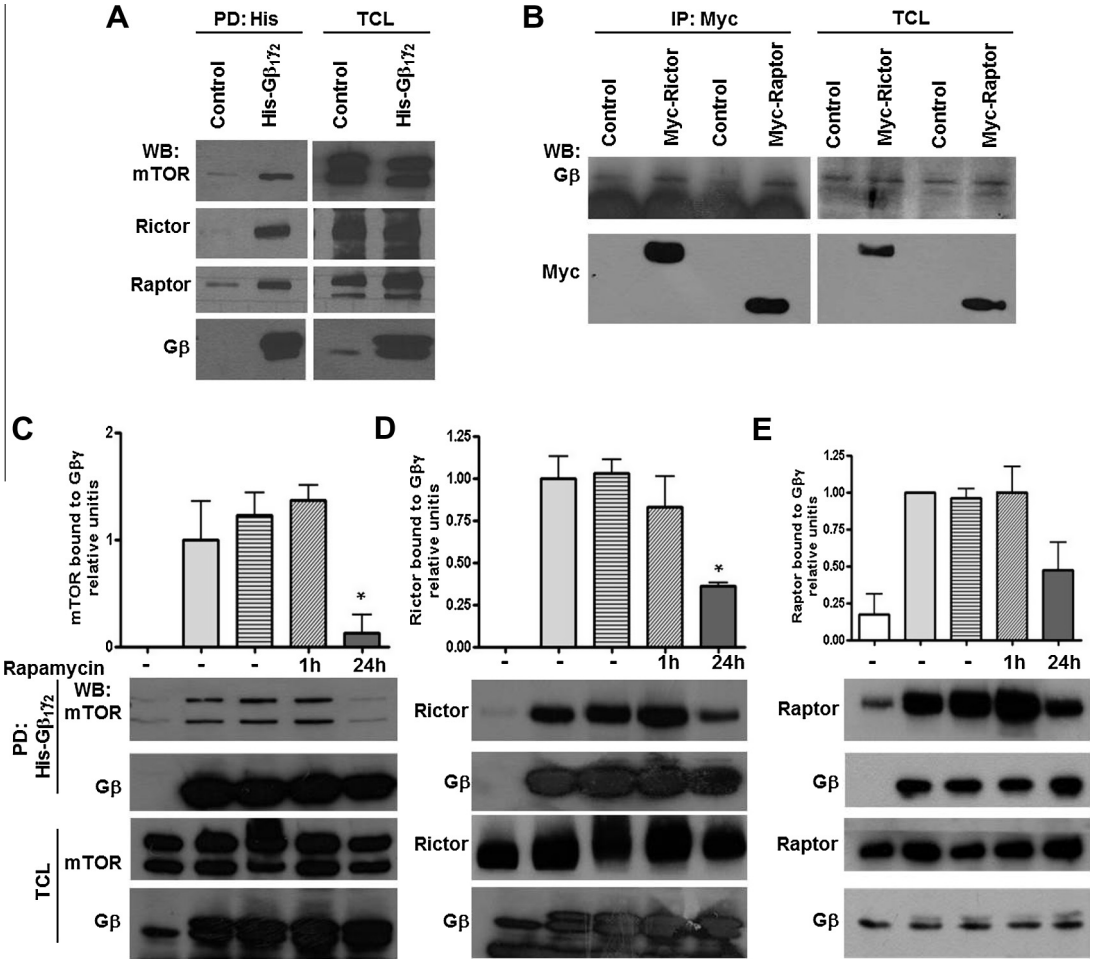

Fig. 2. Gβγ interacts with mTORC1 and mTORC2. (A) Gβγ interacts with endogenous mTORC1 and mTORC2. HEK-293 cells were cotransfected with His-Gβγ and subjected to Talon pulldown to isolate His-Gβγ and proteins bound to it, endogenous mTOR, Raptor and Rictor were detected by Western blotting using the indicated antibodies in both pull downs (PD; left panel) and total cell lysates (TCL; right panel). (B) Gβγ is present in multiprotein complexes containing Rictor or Raptor. Endogenous Gβγ and either Myc-Rictor or Myc-Raptor transfected into HEK293 cells were detected in immunoprecitates (IP:myc; left panel) and total cell lysates (TCL; right panel). (C) Chronic treatment with rapamycin decreases the interaction between Gβγ and mTOR. HEK-293 cells expressing His-Gβγ were incubated with rapamycin (100 ng/ml) for short term (1 h) or long term (24 h). The interaction of Gβγ with mTOR was detected by Western blotting in the His-pull downs. Equivalent experiments were done to assess the effect of rapamycin on the interaction between Gβγ and mTORC2 (detected by the presence of Rictor) (D) or Gβγ and mTORC1 (detected by the presence of Raptor) (E). Graphs represent the mean and standard error of the mean of three independent experiments, $^*p < 0.05$.

interact with each mTOR complex, we explored the presence of endogenous mTOR, Rictor and Raptor in the Gβγ pull down obtained from HEK293T cells transfected with His-Gβγ, as shown in Fig. 2A, both mTOR complexes interacted with Gβγ. We next tested whether endogenous Gβγ interacts with both complexes. As shown in Fig. 2B, Gβγ interacted with mTORC1 and mTORC2. We then tested the effect of rapamycin on the interaction between Gβγ and mTOR. Since it is known that a short term incubation with rapamycin affects the kinase activity of mTOR, whereas a chronic incubation with this inhibitor affects the assembly of mTORC2, we explored the effect of both conditions on the interaction between Gβγ and mTOR. As shown in Fig. 2C, rapamycin incubated for 1 h did not affect the interaction between Gβγ and mTOR, whereas chronic incubation with rapamycin reduced the interaction between these proteins, and also decreased the presence of Rictor and Raptor in the Gβγ pulldowns (Fig. 2D and E, respectively).

3.3. Role of Gβγ in mTORC1 and mTORC2 signaling

Each mTOR complex regulates different cellular activities through the phosphorylation of distinct substrates. mTORC1 phosphorylates S6K and 4EBP-1 and regulates cell growth, whereas mTORC2 regulates cell survival and motility by phosphorylating AKT [18,24,25]. Thus we wanted to determine whether Gβγ could be involved in the activation of mTORC1 and mTORC2. In order to assess this possibility, Gβγ was overexpressed and phosphorylation of S6K and AKT, distinctive substrates of mTORC1 and mTORC2 respectively, was tested. The amount of phospho-S6K and phospho-AKT increased with of Gβγ overexpression (Fig. 3A), an effect that was attenuated by chronic incubation with rapamycin (Fig. 3B). These results correlated with the activation of ERK, a very well characterized readout of Gβγ signaling. In addition, mTOR was activated in the signaling cascade of LPA, which activates endogenous G protein coupled receptors, further confirming the role of mTOR in GPCR signaling.

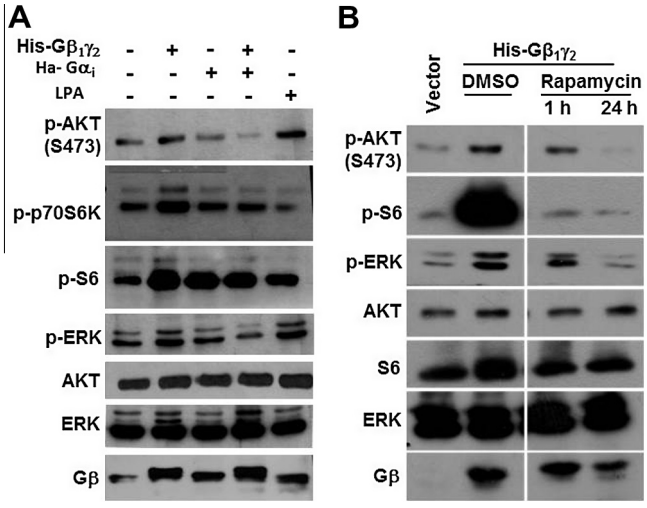

Fig. 3. Gβγ promotes mTORC1 and mTORC2 signaling via a rapamycin sensitive pathway. (A) Gβγ promotes the phosphorylation of AKT at Ser473 (indicative of mTORC2 activation), p70S6K and S6 (indicative of mTORC1 activation) and ERK. HEK-293 cells were transfected with His-Gβγ, HA-Gαi or both, as indicated. Two days after transfection, cells that were starved of serum for 16 h and left unstimulated or stimulated with 10 μM LPA for 5 min (as a positive control). The phosphorylation and expression of the indicated proteins was detected by western blotting in total cell lysates. (B) Gβγ signaling to mTORC1 and mTORC2 was sensitive to rapamycin. Serum starved HEK-293 cells expressing His-Gβγ were incubated with rapamycin (100 ng/ml) for short term (1 h) and long term (24 h), Gβγ-dependent activation of mTORC2, mTORC1 and ERK were detected by western blot using the indicated antibodies.

4. Discussion

We found that endogenous Gβγ interacts with endogenous mTOR. This interaction involves the kinase domain of mTOR. The ability of Gβγ to promote mTORC1-dependent phosphorylation of S6K and mTORC2-dependent phosphorylation of AKT correlates with its interaction with both, mTORC1 and mTORC2, complexes. Interestingly, four out of five existing Gβ isoforms are able to interact with mTOR. Surprisingly, the only Gβγ heterodimer that does not interact with mTOR is the one containing $G\beta_4$. This is unexpected considering that Gβ5, which is the less homologous member of the family, does interact with mTOR. These data suggest that mTOR would not be a prototypic effector of Gβγ.

Gβγ interaction with mTOR does not depend on mTOR kinase activity, at least for the case of rapamycin-sensitive mTORC1, as evidenced by the interaction between these proteins occurring in cells treated with rapamycin for one hour. In contrast, the interaction between Gβγ and mTOR decreased by chronic treatment with rapamycin, suggesting that integrity of mTOR complexes is required for their interaction with Gβγ, consistent with reports showing that assembly of mTOR complexes is reduced by this treatment [31]. Since stimulation of cells with serum affected the interaction of Gβγ with mTOR, a potential role for Gβγ as a regulator of mTOR signaling is either as a transducer to mTOR activation in response to serum or related to a potential ability to restrict a population of mTOR to be responsive to such stimulation. Furthermore, results showing that different mTOR substrates are phosphorylated in response to Gβγ overexpression suggest that an initial interaction between Gβγ and mTOR would be related to the localization of a population of mTOR sensitive to be activated in response to GPCR signaling. Our results are consistent with a model in which the Gβγ/PI3K/AKT/mTOR/P-Rex1 signaling cassette is integrated by multiple interactions and include a novel connection that would contribute to sustain this dynamic complex. This might represent an equivalent system to the observed in *Dictiostelium discoideum*, where the effect of Gβγ on TORC2 signaling partially occurs independently of PI3K [29]. In conclusion, Gβγ interacts with mTOR kinase domain and this correlates with the activation of mTORC1 and mTORC2 downstream of Gβγ.

5. Disclosure

The authors have nothing to disclose.

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