Cellular Signalling 21 (2009) 293–300

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Sphingosine-1-phosphate induced mTOR-activation is mediated by the E3-ubiquitin ligase PAM

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ARTICLE INFO

Article history:
Received 16 July 2008
Received in revised form 10 October 2008
Accepted 23 October 2008
Available online 30 October 2008

Keywords:
PAM
mTOR
Sphingosine-1-phosphate
Rheb
TSC2
Ubiquitin ligase
Guanosine exchange factor
Insulin

ABSTRACT

The signaling pathways that are regulated by sphingosine-1-phosphate (S1P) and mammalian target of rapamycin (mTOR) modulate cell growth, mitogenesis and apoptosis in various cell types and are of major interest for the development of new cancer therapeutics. Previous reports show that S1P can cross-activate the mTOR pathway although the mechanisms that connect both pathways are still unknown. We found that S1P-treatment activates mTOR in several cancer cell lines and primary cells. The activation was independent of ERK, Akt and PI3-kinase, but instead was mediated by the E3 ubiquitin ligase Protein Associated with Myc (PAM). Increased intracellular PAM concentrations facilitated S1P- and insulin-induced mTOR activation as well as p70S6K and 4EBP1 phosphorylation while genetic deletion of PAM decreased S1P- and insulin-induced mTOR activation. PAM activated by facilitating the GDP/GTP-exchange of Rheb which is an activator of mTOR. In conclusion we show that PAM is a novel regulator of the mTOR pathway and that PAM may directly activate Rheb as a guanosine exchange factor (GEF).

1. Introduction

The bioactive sphingolipid metabolite sphingosine-1-phosphate (S1P) is synthesized by phosphorylation of sphingosine by sphingosine kinases (SPHK) in a wide variety of cell types in response to extracellular stimuli. Due to the significant role of S1P in tumor growth, survival, metastasis, immunity and angiogenesis, SPHK, the enzyme that generates S1P, became a major target for anticancer drug development [1–3]. The various cellular actions of S1P are mediated either through the activation of a family of five G-protein coupled receptors ($S1P_{1-5}$) or by acting as an intracellular second messenger [4–6]. S1P-receptors couple to a variety of G-proteins allowing them to regulate different signaling pathways including the inhibition of cAMP signaling, the activation of phosphatidylinositol-3 kinase (PI3K), phospholipase C (PLC), and the activation of the small G-proteins Ras, Rho, and Rac. Previous reports show that S1P can also activate the mammalian target of rapamycin (mTOR) pathway to modulate apoptosis and mitogenesis in osteoblasts, fibroblasts and vascular smooth muscle cells [7–9]. mTOR is a protein kinase that is centrally involved in the control of cancer cell metabolism, cell growth and cell proliferation [10]. Therefore the mTOR pathway has attracted broad clinical interest leading to several ongoing clinical trials investigating the use mTOR inhibitors for the treatment of different cancer types [11,12]. Although in a number of clinical and preclinical studies the combined treatment of S1P receptor agonists and mTOR inhibitors were tested [13–15], surprisingly little is known about the interactions of both pathways [16].

Reports investigating the interaction of both pathways showed that S1P-induced proliferation of fibroblast and vascular smooth muscle cells is mediated by p70 S6 kinase (p70S6K) independently of AKT/protein kinase B and PI3K activation [8,9,17]. However, how the activation of the mTOR pathway by S1P is achieved remains unclear. Here we report that S1P induces in various cancer cell lines as well as in primary cells the activation of the mTOR/p70S6K pathway suggesting that activation of mTOR by S1P represents a common signaling mechanism. As expected the activation was independent of AKT or PI3 kinase and was instead mediated by a novel mTOR activation mechanism that is based on the activation of the small GTPase Rheb by the E3 ubiquitin ligase Protein Associated with Myc (PAM).

2. Materials and methods

2.1. Materials

S1P was purchased from Tocris (Ellisville, MO), FTI-277 from Merck Biosciences (Schwalbach, Germany), anti-TSC2 antibody from Santa Cruz (Santa Cruz, CA), anti-ubiquitin antibodies from Sigma (St. Louis, MO) and Assay Design (Ann Arbor, MI), anti-mTOR, anti-phospho-mTOR (S2448), anti-phospho-p70S6K (T389), anti-phopspho-4EBP1 (T70), and

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doi:10.1016/j.cellsig.2008.10.016

anti-phospho-TSC2 (T1462) antibodies from Cell Signaling (Beverly, MA) and anti-HSP70 antibody from BD Transduction Lab. (Los Angeles, CA). VPC23153 and VPC24191 were from Avanti Polar Lipids (Alabaster, AL). JTE013 from Tocris Bioscience (Ellisville, MO).

2.2. Cell culture

HeLa cells and MCF7 cells (German Resource Centre for Biological Material, DSMZ, Germany) were grown in RPMI 1640 with 10% fetal bovine serum (FCS) and DMEM-Glutamax II with 10% FCS, respectively (all Invitrogen, Carlsbad, CA). Primary embryonic fibroblasts were generated from E16 rats as published previously [18] with the exception that the cells were grown in DMEM medium with 10% FCS.

2.3. DRG isolation and primary cultures

20–30 dorsal root ganglions (DRG) were prepared from adult mice and pooled in ice-cold Hanks' balanced salt solution (HBSS). Then the DRGs were transferred to Neurobasal Medium (Invitrogen, Carlsbad, CA) containing 500 U/ml collagenase (Biochrome AG, Berlin, Germany) and 2.5 U/ml Dispase II (Roche, Mannheim, Germany) and incubated at 37 °C for 2 h. The dissociated tissues were washed twice with Neurobasal Medium containing 10% FCS and once with HBSS. Afterwards the cells were incubated in 0.25% Trypsin/HBSS containing 1 U/ml DNase (Promega, Madison, WI) for 8 min at 37 °C and washed again three times in Neurobasal Medium with 10% FCS. Cells were collected in 2 ml of medium, carefully loaded on top of 5 ml 15% fatty acid free BSA (Sigma, St. Louis, MO)/PBS and centrifuged at 1000 ×*g* for 10 min at room temperature. After discarding the supernatant the isolated cells were collected in Neurobasal Medium containing B27, glutamine and 10% FCS and plated on poly-L-lysine covered dishes. After 2 h the medium was changed to Neurobasal Medium without FCS. After 24 h NGF (0.01 µg/ml) and mitose inhibitor (10 µM uridine, 1 µM cytosin-β-arabino-furanoside, 10 µM 5-fluoro-3-desoxyuridine) was added for 24 h. The neurons were used for experiments after 4–5 days.

2.4. Protein expression and purification

Full length PAM [19,20] and GST-Rheb [21] were purified as previously described. GTP and GDP were removed from GST-Rheb prior use by EDTA treatment as described previously [22]. Dominant negative and constitutively active Rheb constructs were generated by introducing the mutations D60K [23] and Q64L [24], respectively, following the protocol of Gama and Breitwieser [25] using for the dominant negative Rheb the primers phospho-TAAAGACAGCCGGGCAAGATGAATAT and phospho-CAAGTTGAAGATGATATTCTTGTCCA and for the constitutively active phospho-GGGCTAGATGAATATTCTATCTT and phospho-GGCTGTGTCTACAAGTTGAA. The final constructs were sequenced to verify the sequence and the introduction of the mutation.

2.5. In vitro ubiquitination

The assay was performed in 40 mM Tris pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, 25 µM MG132. 50 nM rabbit E1 (Calbiochem, Darmstadt, Germany), 500 nM E2 proteins (Boston Biochem, Cambridge, MA), and 0.15 µg/µl GST-ubiquitin (Sigma, St. Louis, MO). HeLa lysate was incubated in the absence or presence of full length PAM (100 nM) for 75 min at 27 °C. The reaction was terminated with gel loading buffer. HeLa lysates were prepared by sonication of HeLa cells in 50 mM Tris, 1 mM EDTA, 1 mM PMSF, 3 mM benzamidine, 1 µg/ml aprotinin/leupeptin followed by a centrifugation for 5 min at 13,000 ×*g*.

2.6. Immunocytochemistry

To monitor distribution of PAM and TSC2 in HeLa cells, the cell was fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The cells were blocked for 1 h in 3% BSA in PBS and then incubated for 1 h with the anti-TSC2 antibodies followed by incubation with Cy3-labeled goat anti-rabbit antibody (Sigma). Finally FITC-labeled PAM antibody (MelTec, Magdeburg, Germany) was applied for 1 h.

2.7. Western blot analysis

HeLa cells were plated at 200,000 cells/35 mm dishes and serum-starved for 24 h. After the indicated treatment the medium was removed and the cells harvested in boiling 1× Laemmli buffer containing 100 mM sodiumphosphate and 0.2 mM sodium orthovanadate. Signal strength was determined densitometrically using ImageJ software (NIH, USA).

2.8. Immunoprecipitations

HeLa cells were serum-starved overnight and treated as indicated. Co-immunoprecipitations using the TSC2 antibody were done as described previously [26]. For steady state GTPase assays cells were harvested with 1 mM EDTA in PBS, lysed in 1 ml 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1% Nonidet P-40, 5 mM sodium fluoride, 1 mM orthovanadate, 1 mM EDTA, 1 mM PMSF, 3 mM benzamidine, 10 µg/ml aprotinin/leupeptin, 0.25% Na-deoxycholate, incubated for 1 h with anti-TSC2 antibody and precipitated with protein A/G agarose.

2.9. Liposomal delivery of proteins

HeLa cells were plated at 150 000 cells/35 mm dish and serum-starved for 24 h. Purified proteins were diluted in 10 mM Tris to a final concentration of 100 µg/ml (GST or GST-Rheb) or 50 µg/ml (PAM). 175 µl of each stock was used to rehydrate 10 µl BioPorter reagent (Genetherapy Systems, San Diego CA). 815 µl serum-free medium were added and the cells incubated with the mixture for 4 h. The cells were washed twice with serum-free medium, treated as indicated and subjected to western blot analysis. Transfection efficiency was determined using Cy3-labeled antibodies and was 90–100%.

2.10. Steady-state GTPase

The steady-state GTPase activity of GST-Rheb (500 nM) in the presence of indicated purified PAM (30 nM) or immunoprecipitated TSC2 was performed as described previously [27].

2.11. GTP-γ-S-binding

100 nM GST-Rheb was incubated at 25 °C with 100 nM GTP-γ-^{35}S (100 Ci/mmol) for 30 min in the presence of the indicated PAM concentrations in 20 mM Tris pH 8.0, 10 mM $MgCl_2$, 5 mM DTT. Samples were filtered through nitrocellulose filters (Schleicher&Schuell, Dassel, Germany), washed 6 times with 2 ml TMN (20 mM Tris pH 8, 25 mM $MgCl_2$, 100 mM NaCl), dried and analyzed by liquid scintillation counting. To correct for unspecific binding of GTP-γ-^{35}S to PAM, samples with PAM in absence of GST-Rheb were determined and subtracted.

2.12. GDP-dissociation assay

2 µM purified GST-Rheb was loaded for 30 min at 25 °C with 2 µM ^{3}H-GDP (12.4 mCi/mmol) in 25 mM Tris pH 7.4, 50 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA. 150 nM of the ^{3}H-GDP-loaded GST-Rheb were incubated with the indicated PAM concentrations in TMN and treated as described above.

2.13. Data analysis

The statistical analysis was performed using two-tailed Student's *t*-test. *P*-values of less than 0.05 were considered to be significant.