



HAL
open science

Identification of Protor as a novel Rictor-binding component of mTOR complex-2

Laura R Pearce, Xu Huang, Jerome Boudeau, Rafal Pawlowski, Stephan Wullschleger, Maria Deak, Adel Ibrahim, Robert Gourlay, Mark A Magnuson, Dario R Alessi

► To cite this version:

Laura R Pearce, Xu Huang, Jerome Boudeau, Rafal Pawlowski, Stephan Wullschleger, et al.. Identification of Protor as a novel Rictor-binding component of mTOR complex-2. *Biochemical Journal*, 2007, 405 (3), pp.513-522. 10.1042/BJ20070540 . hal-00478790

HAL Id: hal-00478790

<https://hal.science/hal-00478790>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Identification of Protor as a novel Rictor-binding component of mTOR-complex-2

By

Laura R. Pearce*¹, Xu Huang*¹, Jérôme Boudeau¹, Rafał Pawłowski¹, Stephan Wullschleger¹, Maria Deak¹, Adel F.M. Ibrahim¹, Robert Gourlay¹, Mark A. Magnuson² and Dario R. Alessi¹.

1. MRC Protein Phosphorylation Unit, School of Life Sciences, MSI/WTB complex, University of Dundee, Dow Street, Dundee DD1 5EH, Scotland.

2. Department of Molecular Physiology and Biophysics and Centre for Stem Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Correspondence to: LRP (L.r.pearce@dundee.ac.uk).

* **These authors made similar contributions to the study.**

Key Words: Protein kinase, Mass Spectrometry, Cancer.

Abbreviations: CHAPS, 3-(3-cholamidopropyl)diethyl-ammonio-1 propanesulfonate; DTT, Dithiothreitol; GFP, Green fluorescent protein; GST, Glutathione S-transferase; LDS, Lithium dodecyl sulphate; mTOR, mammalian Target Of Rapamycin; mTORC, mTOR complex; PKB, Protein kinase B; Protor, Protein observed with Rictor; Raptor, Regulatory associated protein of mTOR; Rictor, Rapamycin insensitive companion of mTOR; TAP, Tandem affinity purification; TBS, Tris buffered saline.

Short Title: Role of Protor isoforms.

Author's contributions: Laura Pearce undertook most of the experimentation, planned experiments, contributed to writing the paper, and generated the figures; Xu Huang undertook the Rictor immunoprecipitations that led to the discovery of Protor-1, undertook the initial characterisation of this protein and provided helpful discussion; Jérôme Boudeau undertook studies expressing all mTORC2 components to demonstrate that Protor-1 interacts with Rictor rather than other subunits of this complex and provided helpful discussion; Rafał Pawłowski undertook the initial characterisation of Protor-1; Maria Deak and Stephan Wullschleger generated expression constructs and provided helpful discussion; Robert Gourlay undertook the mass spectrometry; Mark Magnuson provided the Rictor knock-out fibroblasts; Dario Alessi conceived the project, helped plan, interpret experiments and write the manuscript.

Abstract

The mTOR protein kinase is an important regulator of cell growth. Two complexes of mTOR have been identified, complex-1, consisting of mTOR:Raptor:mLST8 (termed mTORC1) and complex-2, comprising mTOR:Rictor:mLST8:Sin1 (termed mTORC2). mTORC1 phosphorylates the p70 ribosomal S6 Kinase (S6K) at its hydrophobic motif (Thr389), whilst mTORC2 phosphorylates protein kinase B (PKB) at its hydrophobic motif (Ser473). Here we report that widely expressed isoforms of unstudied proteins termed Protor-1 (PRotein Observed with RicTOR) and Protor-2, interact with Rictor and are components of mTORC2. We demonstrate that immunoprecipitation of Protor-1 or Protor-2, result in the co-immunoprecipitation of other mTORC2 subunits, but not Raptor, a specific component of mTORC1. We show that detergents such as Triton-X100 or n-octylglucoside dissociate mTOR and mLST8 from a complex of Protor-1, Sin1 and Rictor. We also provide evidence that Rictor regulates the expression of Protor-1 and that Protor-1, is not required for the assembly of other mTORC2 subunits into a complex. Protor-1 is a novel Rictor-binding subunit of mTORC2, but further work is required to establish its role.

Introduction

The mammalian Target Of Rapamycin (mTOR) plays a vital role in coupling cell growth to signalling pathways, availability of nutrients and cellular energy supplies [1]. Recent studies in yeast and mammals indicate that mTOR exists in two different complexes which have been termed mTOR Complex-1 (mTORC1) and mTOR Complex-2 (mTORC2) (reviewed in [1, 2]). mTORC1 consists of mTOR, Raptor and mLST8 (previously known as G β L) and is acutely inhibited by the macrolide rapamycin [3-5]. mTORC1 is activated by growth factors via a PI 3-kinase regulated pathway, involving PKB mediated phosphorylation of tuberous sclerosis complex-2 protein and activation of the Rheb GTPase (reviewed in [6]). The activity of mTORC1 is also stimulated by amino acids through a poorly characterised network, which might involve the Vps34 3-phosphatidylinositol-kinase [7, 8] and a sterile-20-related protein kinase termed MAP4K3 [9]. A key cellular substrate for mTORC1 is the ribosomal p70 S6 kinase (S6K), which is phosphorylated by mTORC1 at its hydrophobic motif residue (Thr389) [4, 5], thereby promoting S6K activation by the 3-phosphoinositide dependent protein kinase-1 (PDK1) [10, 11].

mTORC2 consists of mTOR, Rictor (also known as mAVO3), Sin1 (also known as mSin1 or MIP1) and mLST8 [12-17]. Unlike mTORC1, mTORC2 is insensitive to acute rapamycin treatment, although prolonged treatment disrupts mTORC2 assembly in certain cell lines [18]. mTORC2 is thought to be activated by PI 3-kinase through an unknown mechanism, but unlike mTORC1, its activity is not

regulated by amino acids [12, 13, 15, 19]. mTORC2 phosphorylates protein kinase B (PKB) at its hydrophobic motif (Ser473) [19, 20], which together with phosphorylation of the T-loop of PKB (Thr308) by PDK1, is required for maximal activation of PKB [21]. Here we identify a novel Rictor-binding component of mTORC2 as a previously unstudied protein that we have termed Protor-1. Moreover, we demonstrate that an isoform of this protein termed Protor-2 also interacts with mTORC2.

Materials and Methods

Materials. Protein G-Sepharose, calmodulin-Sepharose 4B and glutathione-Sepharose were purchased from Amersham Bioscience; colloidal blue, protease-inhibitor cocktail tablets and precast SDS polyacrylamide Bis-Tris gels and oligofectamine were from Invitrogen; Tween-20, rabbit IgG-agarose and dimethyl pimelimidate were from Sigma, and CHAPS was from Calbiochem. The hexahistidine-tagged TEV protease was expressed in *E. coli* by G. Kular using a construct kindly provided by David Barford and purified using nickel agarose affinity chromatography and gel filtration.

Antibodies. The following antibodies were raised in sheep and affinity purified on the appropriate antigen: anti-mLST8 was raised against the human full length mLST8 protein expressed in *E. coli* (used for immunoblotting), anti-mTOR (residues 2-20 of human mTOR LGTGPAATTAATTSSNVS, used for immunoblotting and immunoprecipitation in 293 cells), anti-Protor-1 was raised against the human full length Protor-1 protein expressed in *E. coli* (used for immunoblotting and immunoprecipitation), anti-Raptor (residues 1-20 of human Raptor MESEMLQSPLLGLGEEDEAD, used for immunoblotting and immunoprecipitation), anti-Rictor (residues 6-20 of human Rictor RGRSLKNLRVRGRND, used for immunoblotting and immunoprecipitation), anti-Sin1 was raised against the human full length Sin1 protein expressed in *E. coli* (used for immunoblotting and immunoprecipitation), anti-SPAK was raised against the human full length SPAK protein expressed in *E. coli* (used for immunoblotting and immunoprecipitation). Anti-mTOR antibody used for immunoblotting of mouse mTOR in mouse fibroblasts was purchased from Santa Cruz Biotechnology (#sc-1549). Anti-Filamin A monoclonal antibody raised against a peptide near the N-terminus was purchased from Santa Cruz Biotechnology (#sc-17749), and was used for immunoblotting and immunoprecipitation. The monoclonal antibody recognising the FLAG epitope tag was purchased from Sigma (#F1804) and secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Pierce.

General methods. Tissue culture, immunoblotting, restriction enzyme digests, DNA ligations, and other recombinant DNA procedures were performed using standard protocols. DNA constructs used for transfection were purified from *E. coli* DH5 α using Qiagen plasmid Mega or Maxi kit according to the manufacturer's protocol and transfection studies were carried out using polyethylenimine as described previously unless otherwise stated [22]. All DNA constructs were verified by DNA sequencing, which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, UK, using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers. Mouse embryonic fibroblasts were cultured with additional non-essential amino acids and 1% sodium pyruvate solution.

Buffers. CHAPS-Lysis buffer contained: 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.3 % (w/v) CHAPS, 1 mM sodium orthovanadate, 10 mM sodium- β -glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.15 M NaCl, 0.1 % (v/v) 2-mercaptoethanol and either 1 mM benzamidine and 0.1 mM phenylmethylsulphonylfluoride or complete proteinase inhibitor cocktail (one tablet/50 ml). Triton-Lysis Buffer was identical to the CHAPS-Lysis Buffer except that 1% (v/v) Triton-X100 replaced the CHAPS detergent. Buffer A contained: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, and 1 mM DTT. Buffer B contained: 50 mM Tris/HCl pH 7.5, 0.15 M NaCl, 0.27 M sucrose, 0.3% (w/v) CHAPS and 1 mM DTT. Buffer C contained: 50 mM Tris/HCl pH 7.5, 0.15 M NaCl, 1 mM MgCl₂, 1 mM imidazole, 2 mM CaCl₂, 0.27 M sucrose and 1 mM DTT. Buffer D contained: 10 mM Tris/HCl pH 7.5, 1 mM imidazole, 20 mM EGTA, and 1 mM DTT. TBS-Tween Buffer contained: 50 mM Tris/HCl pH 7.5, 0.15 M NaCl and 0.1% (v/v) Tween-20.

Plasmids. All IMAGE clones were purchased from GeneService Ltd. A Sin1 cDNA (accession number: NM_001006617) IMAGE clone 2823015 encoding for a splice variant termed mSin1.2 [15]), lacking 36 amino acids between residues 320-356 was obtained. Standard PCR methods were used to create a clone that is identical in sequence to the longest Sin1 isoform (mSin1.1 [15], accession number: NM_001006617), which was subcloned into Strataclone blunt PCR vectors, (Stratagene) and further subcloned as a Bgl2-Not1 fragment into different expression vectors (pGEX6p, Flag-pCMV, pEGB2T). A full-length clone of Protor-1 α (accession number: NM_851850), IMAGE clone 5013578 was obtained, amplified and subcloned into Strataclone blunt PCR vectors and then further subcloned into different expression vectors (pGEX6p, Flag-pCMV, pEGB2T) as a Bgl2-Not1 fragments. This cDNA was utilised to PCR the Protor-1 β (accession number: NP_056181) and Protor-1 γ (accession number: NP_001017530) splice variants. A full-length clone of Protor-2 (accession number: CAE45978) was obtained from

RZPD German Resource Centre for Genome Research (EST DKFZp686N03132). The open reading frame was amplified, the PCR product was subcloned into Strataclone blunt PCR vectors and then further subcloned into different expression vectors (pGEX6p, Flag-pCMV, pEGB2T) as a BamHI-NotI fragment. Full-length cDNA of human mTOR (accession number: NP_004949) was obtained from the IMAGE consortium (40125717). N-terminal Flag and HA tagged forms of mTOR were created by PCR and subcloned as a NotI-NotI fragment into vectors (Flag-pCMV, HA-pCMV pEGB2T). A pRL62-3 construct encoding for mouse Rictor (accession number: AY497009), was kindly provided by M. Hall and R. Loewith (Biozentrum, University of Basel). This construct was used as a PCR template to subclone Rictor into the pSC-A vector (Stratagene). Rictor was subsequently subcloned as a BclI- BclI fragment into several expression vectors (Flag-pCMV, HA-pCMV, pEGB2T and to pEBGFP-C2-TAP [23]). A Human mLST8 coding sequence (accession number: BC020499) was PCR amplified from IMAGE EST clone No: 3903638, cloned into the pGEM-T Easy vector (Promega) and subsequently subcloned as a BamHI-NotI fragment into different expression vectors (pGEX6p, Flag-pCMV5, HA-pCMV5 and pEBG2T). The Raptor cDNA (accession number: NP_065812) was amplified from placenta mRNA (using Superscript III (Invitrogen) kit and primers: 5'-atgctgcaatcgcctcttctgggctg-3' and 5'-ctatctgacacgcttctccaccgagt-3'). The resulting PCR product was ligated into PCR 2.1 TOPO vector (Invitrogen), sequenced and subcloned as an EcoRI-EcoRI or BamHI-NotI fragment into different expression vectors (Flag-pCMV5, HA-pCMV5 and pEBG2T).

Immunoprecipitation of endogenous Rictor. Rictor and pre-immune IgG antibodies were covalently coupled to Protein G-Sepharose in a ratio of 1 mg of antibody to 1 ml of resin using a dimethyl pimelimidate cross-linking procedure [24]. 250 mg of HEK293 suspension cell lysate was pre-cleared by incubation with 0.5 ml Protein G-Sepharose for 30 min at 4 °C. The supernatant was incubated with 0.5 ml of Rictor or pre-immune IgG conjugated Protein G-Sepharose for 1.5 hours at 4°C on a rolling shaker. The immunoprecipitates were washed four times with CHAPS-Lysis Buffer and three times with Buffer A. The resin was resuspended in 0.6 ml of 1/80 diluted NuPAGE-LDS Sample Buffer (# NP007 Invitrogen) for 30 min at room temperature, the resin was removed by filtration through a 0.45 µM Spin-X filter and the eluate concentrated by speed-vac to ~20 µl. DTT to a concentration of 10 mM was added to the sample which was heated for 1 min at 95°C and allowed to cool to room temperature. Iodoacetamide was added to 50 mM final concentration, in order to alkylate Cys residues. After incubation in the dark for 30 min at room temperature, samples were electrophoresed on a precast 4-12% SDS-polyacrylamide gel, which was stained with colloidal blue and photographed. The bands labelled in Figure 1A

were excised, washed and digested with trypsin as described previously [25]. Peptides were analysed by combined MALDI-TOF, MALDI-TOF/TOF MS (matrix-assisted laser-desorption ionization-time-of-flight/time of flight mass spectrometry) analysis on an Applied Biosystems 4700 ToF/ToF Proteomics Analyser using 5 mg/ml alpha cyano cinnamic acid in 10 mM ammonium phosphate as the matrix, or by LC-MS on an Applied Biosystems 4000 Q-TRAP. The Celera Discovery System (Applied Biosystems) human database was searched using the Mascot search algorithm (<http://www.matrixscience.com> [26]).

Generation of stable cell line. HEK293 cells were cultured in 10 cm diameter dishes to 30-50% confluence and transfected with 2 µg of the pEGFP-TAP construct encoding human full-length Rictor using Fugene 6 reagent (Roche) according to the manufacturer's instructions. After 48 h, cells were split to five 10 cm diameter dishes. G418 was added to the medium to a final concentration of 3 mg/ml after another 24 hours allowing cells to recover, and then the medium was changed every 24 hours containing fresh G418 for further antibiotic selection. After 14-20 days, individual surviving colonies expressing moderate levels of GFP-fluorescence were selected and expanded. FACS analysis was also performed to ensure uniform expression of GFP in the selected cell lines. In addition, anti-Rictor immunoblotting analysis of lysed cells was performed to ensure that the expressed protein migrated at the expected molecular mass. For the TAP-purification, we selected the stable cell lines that expressed low levels of GFP-TAP-Rictor to maximize the proportion of purified enzyme that is bound to an endogenous binding partner.

Tandem Affinity Purification. The purification method was adapted from the previously described TAP-purification protocol [23, 27]. For each TAP-purification, we cultured ten 75 ml flasks of confluent cell line, trypsinised and resuspended the cells in 500 ml of HEK293 suspension culture medium (pro-293 DMEM) with 3% serum. Cells were harvested when the cell density reached approx. 3 million cells/ml by centrifugation at 800 rpm, 5min, washed twice with ice-cold phosphate buffered saline and lysed in 30 ml ice-cold CHAPS-Lysis Buffer. The lysates were centrifuged at 26,000 x g for 30 min at 4°C and the supernatant incubated with 0.5 ml rabbit IgG-agarose beads for 1 h at 4°C. The IgG-agarose was washed three times with CHAPS-Lysis Buffer, followed by two washes in Buffer B prior to incubation with 2 ml of Buffer B containing 100 µg of TEV protease for 3.5 hours at 4°C. The cleaved TAP-tagged Rictor protein was eluted with 7.5 ml of Buffer C containing 7.5 µl of 1 M CaCl₂ and incubated with 0.25 ml of rabbit calmodulin-Sepharose equilibrated in Buffer C for 1 hour at 4°C. The calmodulin-Sepharose beads were washed with Buffer C twice and then twice with Buffer D. The samples were eluted from the beads

in LDS Sample Buffer, electrophoresed and analysed following digestion with trypsin and mass spectrometry as described above.

Immunoprecipitation of FLAG-Protor-1 α for mass spectrometry analysis.

Fifteen 10 cm diameter dishes of 293 cells at 30-50% confluence were transiently transfected with 5 μ g FLAG-Protor-1 α DNA per dish. 36 h post-transfection, cells were lysed in 0.5 ml CHAPS-Lysis Buffer, clarified by centrifugation and 60 μ l of anti-FLAG antibody covalently attached to protein G-Sepharose was incubated with 12 mg of cell lysate and incubated for 1 hour at 4°C on a vibrating platform. The immunoprecipitates were washed four times with CHAPS-Lysis Buffer and twice with Buffer A. The resin was resuspended in 60 μ l of 1/8 diluted NuPAGE-LDS Sample Buffer, the eluted proteins concentrated, electrophoresed on a polyacrylamide gel and analysed following digestion with trypsin and mass spectrometry as described above.

Immunoprecipitation of forms of FLAG-Protor for immunoblotting analysis. In order to generate samples for immunoblotting, 3 mg of cell lysate derived from transfected cells was incubated with 10 μ l of anti-Flag antibody covalently attached to protein G-Sepharose for 1 hour at 4°C on a vibrating platform. The immunoprecipitates were washed six times with CHAPS-Lysis Buffer in which 2-mercaptoethanol was omitted, followed by two washes with Buffer A in which DTT was omitted. The immunoprecipitates were resuspended in 30 μ l of SDS sample buffer (not containing DTT) and filtered through a 0.45 μ M Spin-X filter to remove the Sepharose resin. DTT to a concentration of 10 mM was added to the eluted samples. These were then subjected to electrophoresis and immunoblotting analysis as described below.

Immunoprecipitation of endogenous mTOR complexes for immunoblotting analysis. HEK293 cells were lysed in CHAPS-Lysis Buffer. Mouse tissues were homogenised in CHAPS-Lysis Buffer. Debris were removed from cell lysates/tissue extracts by centrifugation at 12,000 x g for 20 min. 1 mg of lysate or tissue extract was pre-cleared by incubating with 5 μ l of Protein G-Sepharose. The lysates or tissue extracts were then incubated with 5 μ l of Protein G-Sepharose conjugated to 5 μ g of the indicated antibodies. Except in the case of Filamin-A all other antibodies were covalently conjugated to Protein G-Sepharose. Immunoprecipitations were carried out for 1 hour at 4°C on a vibrating platform. The immunoprecipitates were washed six times with CHAPS-Lysis Buffer or Triton-Lysis Buffer in which 2-mercaptoethanol was omitted, followed by two washes with Buffer A in which DTT was omitted. The immunoprecipitates were resuspended in 30 μ l of LDS sample buffer (not containing DTT), filtered through a 0.45 μ M Spin-X filter, DTT to a concentration of 10 mM

was added and samples subjected to electrophoresis and immunoblot analysis as described below.

siRNA Transfection. All siRNAs including the control (non-targeting) sequence, were ordered from Dharmacon. The sequences of the strands of the siRNA that were utilised were: Protor-1 (sense: GGACAAGAUUCGCUUCUAUdTdT and antisense: AUAGAAGCGAAUCUUGUCCdTdT); Sin1 (siGENOME SMARTpool siRNA Dharmacon # M-014315-00-0050[14]); Rictor (sense: ACUUGUGAAGAAUCGUAUCdTdT; antisense: GAUACGAUUCUUCACAAGUdTdT [13]) and mTOR (sense: CCCUGCCUUUGUCAUGCCUdTdT, antisense: AGGCAUGACAAAGGCAGGGdTdT [5]). HeLa cells at 80% confluence, were transfected with 65nM siRNA using oligofectamine. After 24hrs media was replaced (without siRNA) and cells were cultured for a further 48hrs before harvesting in CHAPS-lysis buffer.

Immunoblotting. Total cell lysate (20 μ g) or immunoprecipitated samples were heated at 70°C for 5 min in LDS sample buffer, and subjected to polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose membrane. Membranes were blocked for 1 hour in TBS-Tween buffer containing 5% (w/v) skimmed milk. The membranes were probed with 1 μ g/ml of the indicated antibodies in TBS-Tween containing 5% (w/v) skimmed milk for 16 hours at 4°C. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent.

Results

Identification of Protor-1 as a Rictor associated protein. In an attempt to characterise novel mTORC2 interacting proteins, we undertook an analysis of proteins that were co-immunoprecipitated with either endogenous Rictor (Fig 1A) or Tandem Affinity Purification (TAP) tagged Rictor that had been stably expressed in 293 cells (Fig 1B). In both purifications, Rictor was a dominant coomassie-staining band migrating at the expected molecular weight of ~180 kDa. As expected, the previously described mTORC2 components mTOR (~260 kDa) [1], mLST8 (~37 kDa) [3, 28] as well as the recently discovered Sin1 (~70 kDa) [14-16] were also associated with Rictor in these studies. Only one other ~42 kDa protein of unknown function (Swiss-Prot accession Number. NP_851850), that we have named Protor-1 (PRotein Observed with RicTOR), was also present in both preparations of Rictor (Fig 1, Supplementary Tables 1 & 2). In one study the gene encoding this protein was called Proline Rich Repeat protein-5 [29]. We also observed that stimulation of 293

cells with IGF-1, did not alter the amounts of Protor-1 or mTOR, Sin1, and mLST8 associated with Rictor (Fig 1).

Protor-1 mRNA is reportedly expressed as three splice variants [29], that we have termed Protor-1 α (residues 1-388, accession Number NP_851850), Protor-1 β (residues 10-388, accession number NP_056181) and Protor-1 γ (residues 97-388, accession number NP_001017530). Protor-1 possesses no recognisable functional domains and has not been studied previously at the level of the protein. The apparent molecular weight with which the Protor-1 protein migrated on an SDS-polyacrylamide gel (~42 kDa) (Fig 1) and mass spectrometry analysis of the peptide sequences, suggested that the splice variant of Protor associated with Rictor in the studies presented in Figure 1, was either Protor-1 α or Protor-1 β , that only differ by the presence of 9 additional residues at the N-terminal (which we failed to detect in the mass spectrometry analysis, Fig 1, Supplementary Tables 1 & 2). Database analysis suggested that there is a second isoform of human Protor, encoded by an unstudied gene that we have termed Protor-2 (accession number CAE45978). Protor-2 encodes a protein of 368 residues displaying 39% identity in amino acid sequence to Protor-1 α (Supplementary Fig 1). The highest region of conservation between Protor-1 and Protor-2 encompasses residues 42-225 of Protor-1 α (Supplementary Fig 1).

A number of other proteins were associated with immunoprecipitated endogenous Rictor (Fig 1A), which were not identified in the purification of the stably-expressed TAP-tagged Rictor (Fig 1, Supplementary Table 1 & 2). Further investigation is required to determine whether any of these proteins interact with Rictor or whether these were non-specifically immunoprecipitated with the Rictor antibody utilised. Two of these proteins, MLEL1 and CDYL that we have tested thus far, failed to interact when co-expressed with Rictor in 293 cells, (LRP, data not shown). We also found that 3 isoforms of 14-3-3 interacted with TAP-Rictor (Fig 1B and Supplementary Table 2), but these adaptor proteins were not detected in the mass spectrometry analysis of the endogenous Rictor immunoprecipitates (Fig 1A and Supplementary Table 1). To our knowledge, neither Rictor nor other components of mTOR complexes have been identified in large-scale proteomic analysis of 14-3-3 interacting proteins.

Protor-1 interacts with mTORC2. We next expressed Flag-Protor-1 α in 293 cells and investigated by mass spectrometry, which endogenously expressed proteins co-immunoprecipitated with it. Consistent with the notion that Protor-1 might comprise a component of mTORC2, mTOR, Rictor, Sin1, were readily detected in the Flag-Protor-1 α immunoprecipitates (Fig 2A). Although mass spectrometry analysis did not detect mLST8 within the Flag-Protor-1 α immunoprecipitates, its presence was confirmed by immunoblotting analysis (Fig 2D). Raptor was not detected in

immunoprecipitates of Flag-Protor-1 α , by either mass spectrometry (Fig 2A) or immunoblotting (Fig 2D). We also co-expressed Flag-Protor-1 α with GST-Rictor or six other GST fusion proteins in 293 cells and observed that Protor-1 α only interacted with Rictor, suggesting that the interaction was specific (Supplementary Fig 2).

Forms of Protor that interact with mTORC2. We next investigated whether Protor-1 splice variants as well as Protor-2 were capable of interacting with endogenous mTORC2 components when overexpressed in 293 cells. The results of these studies demonstrate that Protor-1 β and Protor-2 bind to mTORC2 components to the same extent as Protor-1 α (Fig 2D). Interestingly, Protor-1 γ that lacks the N-terminal part of the region conserved between Protor-1 and Protor-2 (Supplementary Fig 1), failed to interact with any component of mTORC2, despite being expressed at the same level as Protor-2 in 293 cells (Fig 2D).

Endogenous Protor-1 interacts with mTORC2 components. Using an antibody raised against Protor-1, we demonstrate that immunoprecipitation of endogenous Protor-1 in 293 cells results in the co-immunoprecipitation of endogenous mTOR, Rictor, Sin1 and mLST8 (Fig 3A). Furthermore, Protor-1 was clearly detected within immunoprecipitates of endogenous mTOR, Rictor and Sin1 (Fig 3A). Consistent with the view that Protor-1 associates with mTORC2, rather than mTORC1, Raptor was not detected within immunoprecipitates of endogenous Protor-1. As expected, Raptor was detected within immunoprecipitates of mTOR, but not within immunoprecipitates of Rictor or Sin1 (Fig 3A). We noticed that the cytoskeletal protein Filamin-A, co-immunoprecipitated with Flag-Protor-1 α (Fig 2A). However, as we have observed Filamin-A to be a contaminant of other Flag-immunoprecipitations we have undertaken (DRA data not shown), we were concerned that this was a non-specific interaction. Consistent with this, we failed to observe co-immunoprecipitation of endogenous forms of Filamin-A with either Protor-1 α or other mTORC2 components (Fig 3A).

Effects of detergent on the association of Protor-1 with mTORC2 components. Previous studies demonstrated that the integrity of the mTORC2 is disrupted when cells are lysed with 1% (v/v) Triton-X100 detergent, but not with 0.3% (w/v) CHAPS [13]. In the previous experiments undertaken in this study to demonstrate association of Protor-1 with mTORC2, 0.3% (w/v) CHAPS detergent was employed. To investigate how detergents affected the association of Protor-1 with mTORC2, we lysed 293 cells either in the absence of detergent or in the presence of one of four different detergents. None of the detergents employed affected the ability of Protor-1 to associate with Rictor and Sin1, suggesting that the interaction between these proteins is detergent insensitive. Strikingly, when cells were lysed with Triton-X100 or n-octylglucoside, neither mTOR or mLST8 associated with Protor-1 (Fig 3B).

When cells were lysed with Tween, similar association of Protor-1 with mTOR and mLST8 was observed as we found with CHAPS (Fig 3B). The highest levels of mTOR and mLST8 associated with Protor-1 were seen when cells were lysed with no detergent.

Protor-1 interacts specifically with Rictor. In order to establish which components of the mTORC2 Protor-1 α interacts with, mTORC2 subunits and Protor-1 were co-expressed in 293 cells. Immunoprecipitation of Protor-1 α resulted in the co-immunoprecipitation of mTOR, Rictor and Sin1 as expected (Fig 4A). Omitting individual subunits suggested that Protor-1 α , is principally binding to mTORC2 by virtue of its capability to bind Rictor, as in the absence of Rictor, Protor-1 α failed to interact with co-expressed mTOR or Sin1. In the absence of mTOR, Protor-1 still associated with Rictor and Sin1 (Fig 4A, middle panel). In the absence of Sin1, Protor-1 still interacted with Rictor. Consistent with previous reports, mTOR fails to interact with Rictor in the absence of Sin1, as Sin1 is required for the stable binding of Rictor to mTOR [14-16] (Fig 4A, middle panel). Further evidence that Protor-1 does not interact with Sin1, is that in the absence of Rictor, immunoprecipitation of Sin1 did not result in the co-immunoprecipitation of Protor-1 (Fig 4A, lower panel).

Rictor regulates the expression of Protor-1 and Sin1. To further investigate the importance of Rictor in enabling Protor-1 to interact with mTORC2 components, we utilised Rictor^{-/-} knock-out mouse embryonic fibroblast cells that have recently been generated [30]. Strikingly, both Protor-1 and Sin1 expression was vastly decreased in the absence of Rictor (Fig 4B), suggesting that the stability and/or expression of Protor-1 and Sin1 are dependent upon the presence of Rictor.

Knock-down of Protor-1 does not affect association of other mTORC2 components. To investigate the effect of knock-down of Protor-1 on the expression and ability of other mTORC2 subunits to interact with each other, HeLa cells were transfected with siRNA targetting individual mTORC2 components. The siRNA treatments reduced expression of each of the targetted subunit by 80-90% (Fig 4C, upper panel). Knock-down of Protor-1, did not affect the levels of mTOR, Sin1, mLST8 or Rictor in cell extracts. Moreover, immunoprecipitation of either mTOR, Rictor or Sin1 in cells depleted of Protor-1, revealed that the depletion of Protor-1, did not affect the ability of the other mTORC2 components to form a complex (Fig 4C, lower panel). In contrast, knock-down of other mTORC2 components had significant effects on the expression of other subunits. For example, knock-down of mTOR reduced the expression of mLST8, lowering Rictor also decreased expression of Protor-1 and Sin1 and knock-down of Sin1, moderately reduced expression of mTOR and Rictor.

Discussion

In this study we have demonstrated in several ways that Protor-1 comprises a novel component of mTORC2. Our results suggest that Protor-1 binds specifically to the Rictor subunit of the complex, explaining why Protor-1 does not interact with the Raptor-containing mTORC1, which is devoid of Rictor. Based on our observations and the work undertaken by others, we summarise in Fig 5 the proposed subunit organisation of mTORC2. Our data also suggest that in the presence of detergents such as Triton-X100 or n-octylglucoside, the interaction between Rictor and mTOR is disrupted, but the interactions between Protor-1, Rictor and Sin1 are maintained (Fig 3B). We find that the association of mTORC2 subunits is best preserved when cells are lysed with no detergent (Fig 3B). We would recommend in future studies when mTORC2 is isolated from cells or tissues that all detergents are omitted from the lysis procedure.

Our findings also suggest that Rictor controls the expression and/or stability of Protor-1, as in Rictor^{-/-} cells, expression of Protor-1 and Sin1 is markedly reduced (Fig 4B). Reduction in Sin1 protein levels in Rictor^{-/-} cells was also recently reported [14]. This observation further emphasises the link between the Protor and Rictor proteins.

Further work is required to investigate the mechanism by which Rictor controls the expression and/or stability of Protor-1. It is possible that Rictor ensures that expression of Protor-1 and Sin1 are maintained at similar stoichiometric levels to Rictor so that there is not an excess of these subunits. SiRNA knock-down of mTOR, Rictor and Sin1 has moderate to significant effects on the expression of several other mTORC2 subunits (Fig 4C). Similar observations have been made for other protein kinases and their interacting subunits, for example in cells lacking the LKB1 protein kinase, expression of its regulatory STRAD subunit is also hugely decreased [31].

Protor-1 as well as Protor-2 possesses no recognisable functional domains or motifs within their sequence. Hence it is difficult to predict what their physiological role(s) might be, whether they possess enzymatic activity and how they might influence mTORC2. We have attempted siRNA experiments to reduce expression of Protor-1, Sin1, Rictor and mTOR in HeLa cells to investigate how this affects Ser473 phosphorylation of PKB, the proposed target of mTORC2. Although we were able to reduce expression of these components by 80-90% (Fig 4C), this reduction of protein was not sufficient to significantly reduce phosphorylation of PKB at Ser473 (LRP, data not shown). Sabatini and colleagues have elegantly demonstrated that it is essential to completely ablate expression of mTOR and Rictor in order to reduce phosphorylation of Ser473 of PKB[19], conditions that we have so far been unable to recapitulate in our laboratory. We are currently generating Protor knock-out mice to address the physiological roles of this subunit in regulating mTORC2 function.

Sequence alignments of Protor-1 and Protor-2 and Protor-1 homologues found in other vertebrates (Supplementary Fig 3), also highlight that the N-terminal region of Protor encompassing residues 42 to 225 of human Protor-1 is the most highly conserved and may therefore form a functional domain. Protor-1 γ that lacks the first 96 residues, hence part of the conserved domain, was not capable of binding Rictor (Fig 2D). Further work is required to delineate the residues of Protor isoforms and Rictor that directly interact with each other, but it is likely that the Rictor-binding domain is within the conserved region on Protor. In addition, it remains to be investigated whether Protor-2 plays the same role as Protor-1. In our mass spectrometry studies we failed to detect association of Protor-2 with endogenous Rictor or TAP-Rictor, however in the absence of a specific anti-Protor-2 antibody, it is currently unknown whether Protor-2 is expressed in the 293 cells utilised for our experiments.

Analysis of the tissue distribution patterns of mRNA expression of Protor-1 and Protor-2 in humans using the NBI Unigene database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene>), suggests that these proteins, similar to the other known components of mTOR complexes, are widely expressed in all tissues. Northern blot analysis of Protor-1 mRNA in 12 human tissues also supported this conclusion [29]. We have analysed the expression levels of the Protor-1 protein in nine different mouse tissue extracts and found that Protor-1 was broadly expressed, with highest levels in adipose, kidney, spleen and testis and lowest levels in skeletal muscle (Supplementary Fig 4). In the only previous study in which the Protor-1 gene was investigated, it was reported that Protor-1 mRNA was over-expressed in 13 out of 16 colorectal tumours studied [29]. It was also reported that in human metastatic breast cancer, Protor-1 mRNA was over-expressed in 2 out of 8 tumours whilst being down-regulated in 5 out of the 8 cancers [29]. No somatic mutations in the exons encoding for the Protor-1 gene in human breast or colorectal tumours have yet been identified.

Surprisingly, we have not been able to detect any obvious homologues of Protor-1 or Protor-2 in lower eukaryotic species including yeast and *Drosophila* frequently used for genetic analysis of mTOR pathways. In *S.cerevisiae*, two TOR complexes termed TORC1 and TORC2, believed to comprise functional homologues of mTORC1 and mTORC2, have been characterised [3, 32]. *S.cerevisiae* homologues of Rictor (AVO3), Sin1 (AVO1) and mLST8 (LST8) have been identified in TORC2 isolated from yeast extracts [3, 32]. Two other *S.cerevisiae* proteins, namely AVO2 [3] and BIT61 [33] associate with purified TORC2. To our knowledge, no homologues of these proteins have yet been reported in mammals, and neither do

AVO2 or BIT61 possess evident identity to Protor-1/Protor-2. A clear homologue of Protor is found in frogs and fish (Supplementary Fig 3).

In conclusion, we have identified Protor-1 and potentially Protor-2 as novel Rictor-binding components of mTORC2. Further work is required to define the role that Protor plays in regulating the functions of mTORC2. In particular it would be crucial to define whether Protor-1 is required to enable mTOR complexes to couple cell growth to the many signalling networks and environmental cues that mTOR complexes respond to [1].

Acknowledgements

We thank the Sequencing Service (School of Life Sciences, University of Dundee, Scotland) for DNA sequencing, the Post Genomics and Molecular Interactions Centre for Mass Spectrometry facilities (School of Life Sciences, University of Dundee, Scotland) and the protein production and antibody purification teams [Division of Signal Transduction Therapy (DSTT), University of Dundee] co-ordinated by Hilary McLauchlan and James Hastie for generation and purification of antibodies. LRP is supported by a UK MRC studentship. We thank the Association for International Cancer Research, Diabetes UK, the Medical Research Council, the Moffat Charitable Trust and the pharmaceutical companies supporting the Division of Signal Transduction Therapy Unit (AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck & Co. Inc, Merck KgaA and Pfizer) for financial support.

References

- 1 Wullschleger, S., Loewith, R. and Hall, M. N. (2006) TOR signaling in growth and metabolism. *Cell* **124**, 471-484
- 2 Sarbassov, D. D., Ali, S. M. and Sabatini, D. M. (2005) Growing roles for the mTOR pathway. *Curr. Opin. Cell. Biol.* **17**, 596-603
- 3 Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J. L., Bonenfant, D., Oppliger, W., Jenoe, P. and Hall, M. N. (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol. Cell* **10**, 457-468
- 4 Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J. and Yonezawa, K. (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* **110**, 177-189.
- 5 Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., Tempst, P. and Sabatini, D. M. (2002) mTOR interacts with Raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**, 163-175
- 6 Li, Y., Corradetti, M. N., Inoki, K. and Guan, K. L. (2004) TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem. Sci.* **29**, 32-38

- 7 Byfield, M. P., Murray, J. T. and Backer, J. M. (2005) hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *J. Biol. Chem.* **280**, 33076-33082
- 8 Nobukuni, T., Joaquin, M., Rocco, M., Dann, S. G., Kim, S. Y., Gulati, P., Byfield, M. P., Backer, J. M., Natt, F., Bos, J. L., Zwartkruis, F. J. and Thomas, G. (2005) Amino acids mediate mTOR/Raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 14238-14243
- 9 Findlay, G. M., Yan, L., Procter, J., Mieulet, V. and Lamb, R. F. (2007) A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. *Biochem. J.*
- 10 Biondi, R. M., Kieloch, A., Currie, R. A., Deak, M. and Alessi, D. R. (2001) The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO J.* **20**, 4380-4390.
- 11 Collins, B. J., Deak, M., Arthur, J. S., Armit, L. J. and Alessi, D. R. (2003) In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *EMBO J.* **22**, 4202-4211.
- 12 Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M. A., Hall, A. and Hall, M. N. (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat. Cell. Biol.* **6**, 1122-1128
- 13 Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P. and Sabatini, D. M. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and Raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.* **14**, 1296-1302
- 14 Yang, Q., Inoki, K., Ikenoue, T. and Guan, K. L. (2006) Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes Dev.* **20**, 2820-2832
- 15 Frias, M. A., Thoreen, C. C., Jaffe, J. D., Schroder, W., Sculley, T., Carr, S. A. and Sabatini, D. M. (2006) mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr. Biol.* **16**, 1865-1870
- 16 Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S. Y., Huang, Q., Qin, J. and Su, B. (2006) SIN1/MIP1 maintains Rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* **127**, 125-137
- 17 Guertin, D. A., Stevens, D. M., Thoreen, C. C., Burds, A. A., Kalaany, N. Y., Moffat, J., Brown, M., Fitzgerald, K. J. and Sabatini, D. M. (2006) Ablation in mice of the mTORC components Raptor, Rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC α , but not S6K1. *Dev. Cell* **11**, 859-871
- 18 Sarbassov, D. D., Ali, S. M., Sengupta, S., Sheen, J. H., Hsu, P. P., Bagley, A. F., Markhard, A. L. and Sabatini, D. M. (2006) Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol. Cell* **22**, 159-168
- 19 Sarbassov, D. D., Guertin, D. A., Ali, S. M. and Sabatini, D. M. (2005) Phosphorylation and regulation of Akt/PKB by the Rictor-mTOR complex. *Science* **307**, 1098-1101
- 20 Hresko, R. C. and Mueckler, M. (2005) mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. *J. Biol. Chem.* **280**, 40406-40416

- 21 Mora, A., Komander, D., Van Aalten, D. M. and Alessi, D. R. (2004) PDK1, the master regulator of AGC kinase signal transduction. *Semin. Cell. Dev. Biol.* **15**, 161-170
- 22 Durocher, Y., Perret, S. and Kamen, A. (2002) High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res.* **30**, E9
- 23 Al-Hakim, A. K., Goransson, O., Deak, M., Toth, R., Campbell, D. G., Morrice, N. A., Prescott, A. R. and Alessi, D. R. (2005) 14-3-3 cooperates with LKB1 to regulate the activity and localization of QSK and SIK. *J. Cell Sci.* **118**, 5661-5673
- 24 Harlow, E. and Lane, D. (1988) *Antibodies a Laboratory Manual*. Cold Spring Harbor Laboratory Publications, New York
- 25 Woods, Y. L., Rena, G., Morrice, N., Barthel, A., Becker, W., Guo, S., Unterman, T. G. and Cohen, P. (2001) The kinase DYRK1A phosphorylates the transcription factor FKHR at Ser329 in vitro, a novel in vivo phosphorylation site. *Biochem. J.* **355**, 597-607
- 26 Perkins, D. N., Pappin, D. J., Creasy, D. M. and Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**, 3551-3567
- 27 Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Seraphin, B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* **17**, 1030-1032
- 28 Kim, D. H., Sarbassov, D. D., Ali, S. M., Latek, R. R., Guntur, K. V., Erdjument-Bromage, H., Tempst, P. and Sabatini, D. M. (2003) GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between Raptor and mTOR. *Mol. Cell* **11**, 895-904
- 29 Johnstone, C. N., Castellvi-Bel, S., Chang, L. M., Sung, R. K., Bowser, M. J., Pique, J. M., Castells, A. and Rustgi, A. K. (2005) PRR5 encodes a conserved proline-rich protein predominant in kidney: analysis of genomic organization, expression, and mutation status in breast and colorectal carcinomas. *Genomics* **85**, 338-351
- 30 Shiota, C., Woo, J. T., Lindner, J., Shelton, K. D. and Magnuson, M. A. (2006) Multiallelic disruption of the Rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. *Dev. Cell* **11**, 583-589
- 31 Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R. and Hardie, D. G. (2003) Complexes between the LKB1 tumor suppressor, STRADalpha/beta and MO25alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J. Biol.* **2**, 28
- 32 Wullschleger, S., Loewith, R., Oppliger, W. and Hall, M. N. (2005) Molecular organization of target of rapamycin complex 2. *J. Biol. Chem.* **280**, 30697-30704
- 33 Wedaman, K. P., Reinke, A., Anderson, S., Yates, J., 3rd, McCaffery, J. M. and Powers, T. (2003) Tor kinases are in distinct membrane-associated protein complexes in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **14**, 1204-1220

Figure Legends

Figure 1. Identification of Protor-1. (A) 293 Cells were deprived of serum overnight and left untreated or stimulated with IGF-1 (50 ng/ml) for 30 min. 293 cell extracts were subjected to immunoprecipitation with Rictor or pre-immune IgG antibody. The immunoprecipitates were electrophoresed on a polyacrylamide gel and the protein bands visualised following colloidal blue staining. Each band visualised in the immunoprecipitate was given the indicated number, excised together with the equivalent region of the pre-immune immunoprecipitate, digested with trypsin, analysed by mass spectrometry and results obtained are shown in Supplementary Table 1. The bands identified as Protor-1 α and/or Protor-1 β and other mTORC2 components are indicated with an arrow and were not detected within the control immunoprecipitate undertaken with pre-immune IgG antibody. The molecular weight of the indicated proteins is shown in brackets and asterisks denote mTORC2 components. (B) 293 cells stably expressing TAP-Rictor were treated in the presence or absence of IGF-1 as in (A). TAP-Rictor was affinity purified, electrophoresed on a polyacrylamide gel, the protein bands visualized following colloidal blue staining, labelled and analysed as in (A) and the results tabulated in Supplementary Table 2.

Figure 2. Protor-1 binds mTORC2.(A) 293 cells were transfected with a DNA construct encoding FLAG-Protor-1 α . 36 h post-transfection, cells were lysed, the FLAG-Protor-1 α immunoprecipitated, and electrophoresed on a polyacrylamide gel. The major protein bands were visualised following colloidal blue staining and labelled as indicated. The molecular weight of the indicated proteins is shown in brackets and asterisks denote mTORC2 components. (B) The labelled colloidal blue-stained bands identified in (A) were excised from the gel, digested with trypsin and their identities determined by tryptic peptide mass-spectral fingerprint as described in the experimental procedures. † indicates Mascot protein score where a value >67 is considered significant (P<0.05). Abbreviation, N.P.D. is No significant Protein identity Determined. (C) Schematic representation of the Protor-1 isoforms and Protor-2. The conserved region is shaded and numbering of residues is based on human sequence. For a sequence alignment of these proteins, see Supplementary Fig 1. (D) As in (A) except that 293 cells were transfected with the indicated forms of Protor and a control Flag-epitope tagged protein (TD2 accession number: NM_014779). Immunoprecipitates were immunoblotted with the indicated antibodies. Similar results were obtained in 3 separate experiments.

Figure 3. Endogenous Protor-1 binds to mTORC2. (A) 293 cell lysates were subjected to immunoprecipitation with the indicated antibodies raised against different mTORC2 components as well as the SPAK protein kinase as a control that would not be expected to bind mTORC2 and Filamin A detected within the FLAG-Protor-1 α immunoprecipitate shown in Figure 2A. Immunoprecipitates were immunoblotted with the indicated antibodies. Similar results were obtained in 3 separate experiments. The molecular weight of the indicated proteins is shown in brackets. (B) As in (A) except that cells were lysed in a buffer containing either no detergents or the concentrations of the indicated detergents.

Figure 4. Rictor interacts with Protor-1 and regulates its expression. (A) 293 cells were co-transfected with the indicated DNA constructs encoding the indicated mTORC2 components. 36h post-transfection, cells were lysed, and Protor-1 and Sin1 immunoprecipitated. The cell lysates (upper panel), the Protor-1 immunoprecipitates (middle panel) and Sin1 immunoprecipitates (lower panel) were immunoblotted with the indicated antibodies. Similar results were obtained in 2 separate experiments. The molecular weight of the indicated proteins is shown in brackets. (B) Cell lysates derived from control wild type Rictor $+/+$ and knock-out Rictor $-/-$ mouse embryonic fibroblast cells [30], were subjected to immunoblot analysis with the indicated antibodies. To confirm marked reduction in Protor-1 expression, endogenous Protor-1 was immunoprecipitated and immunoblotted (lower panel). (C) HeLa cells were transfected with the indicated siRNAs (sequences in materials and methods). 72hrs post-transfection, cells were lysed and cell lysates (upper panel) and the immunoprecipitates (lower panel) were immunoblotted with the indicated antibodies. Similar results were obtained in two separate experiments. The molecular weight of the indicated proteins is shown in brackets.

Figure 5. Model of the subunit organisation of mTORC2. The dotted line indicates the detergent sensitive interaction between mTOR/mLST8 and Rictor/Sin1/Protor. ? indicates that direct interaction of these subunits is uncertain.

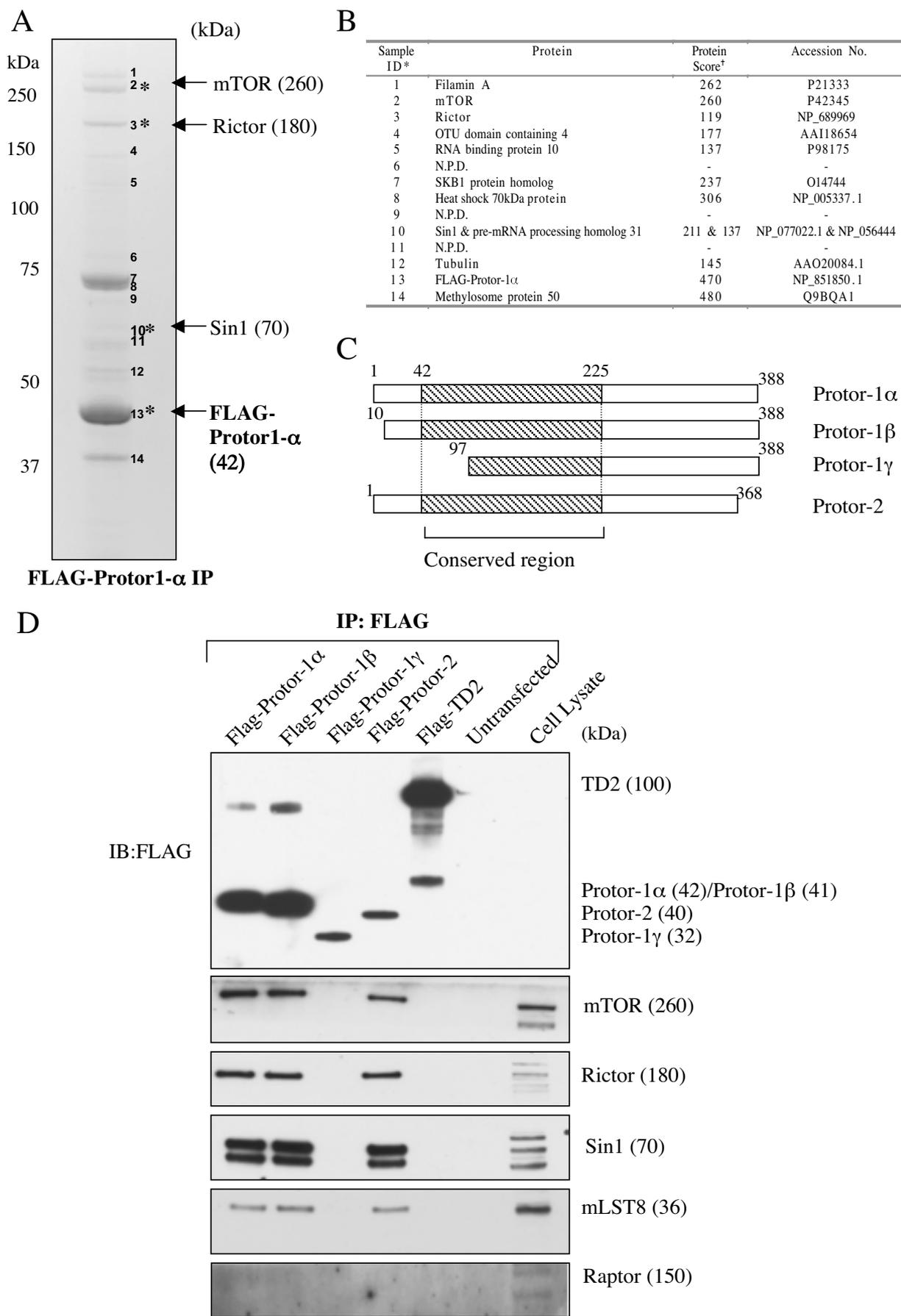
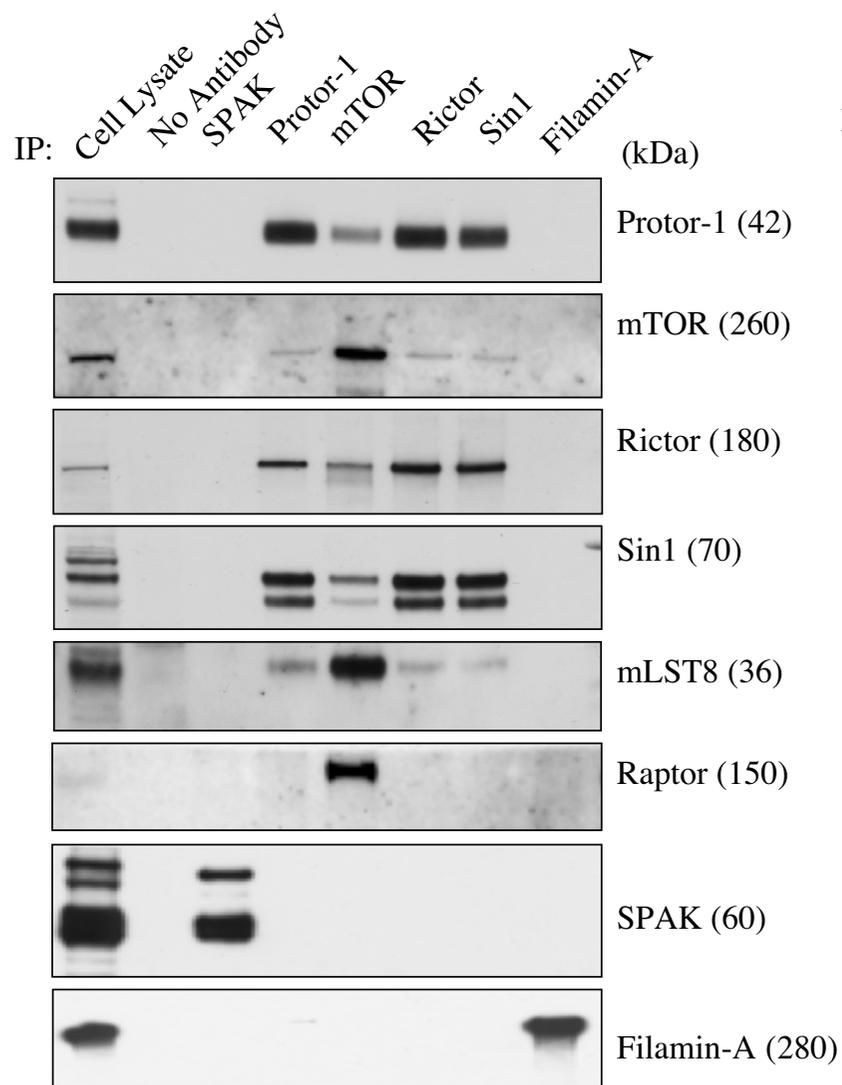


FIGURE 2

A



B

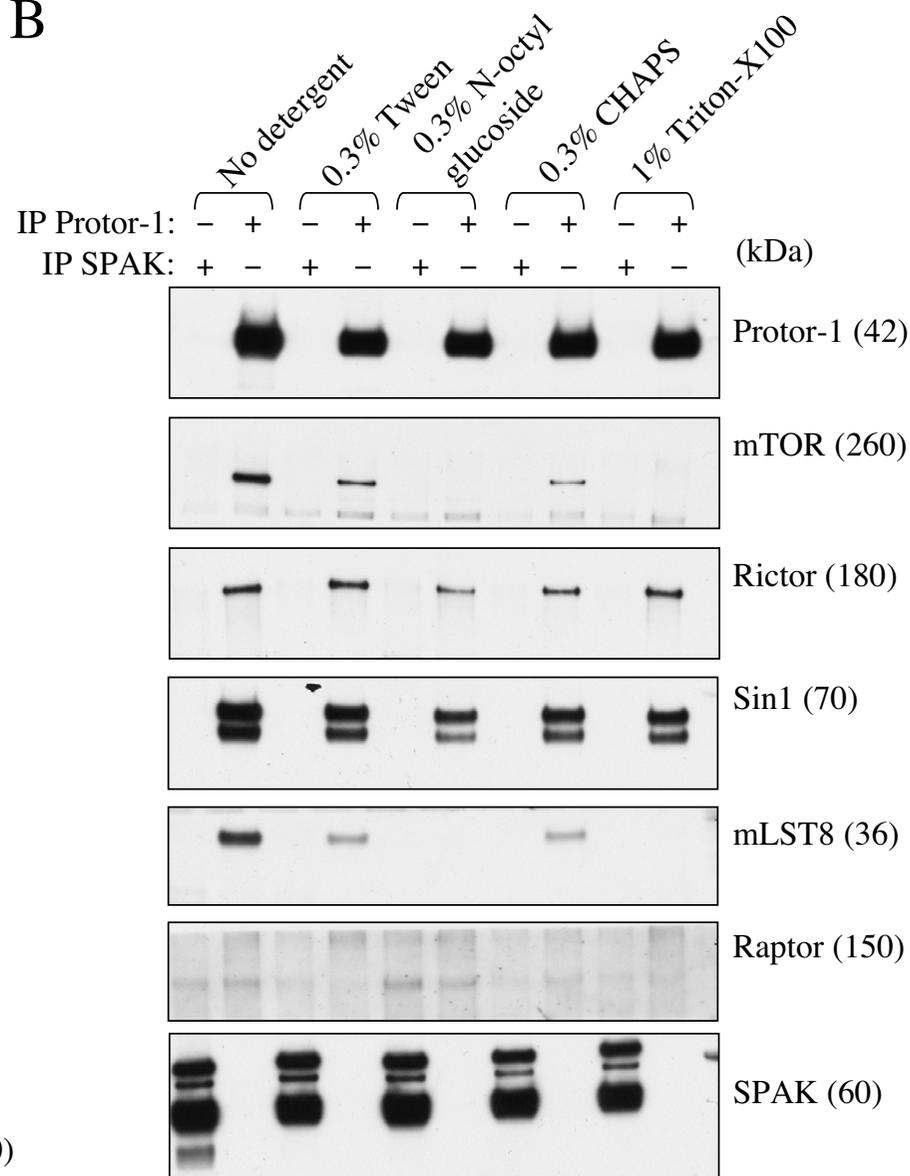


FIGURE 3

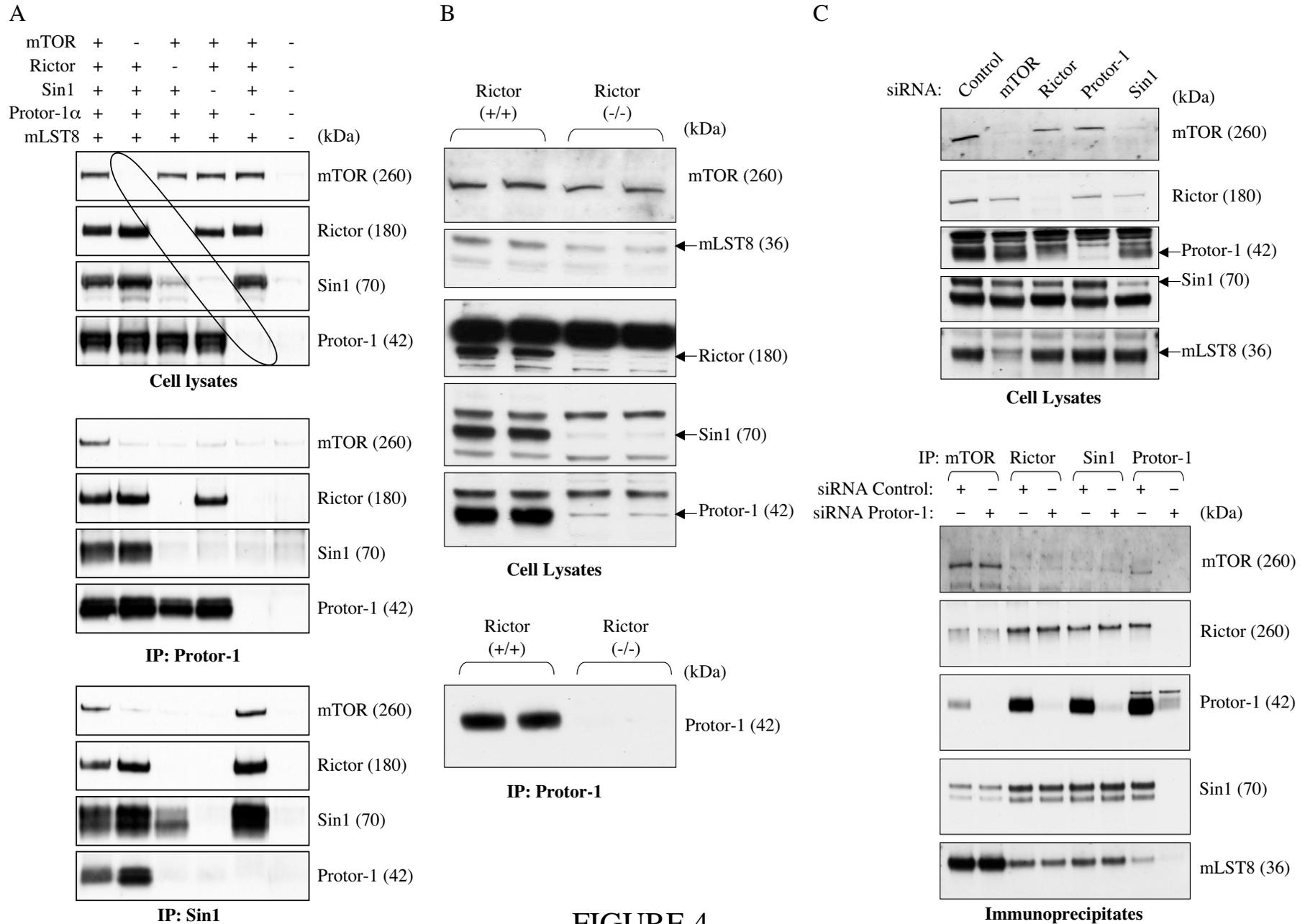


FIGURE 4

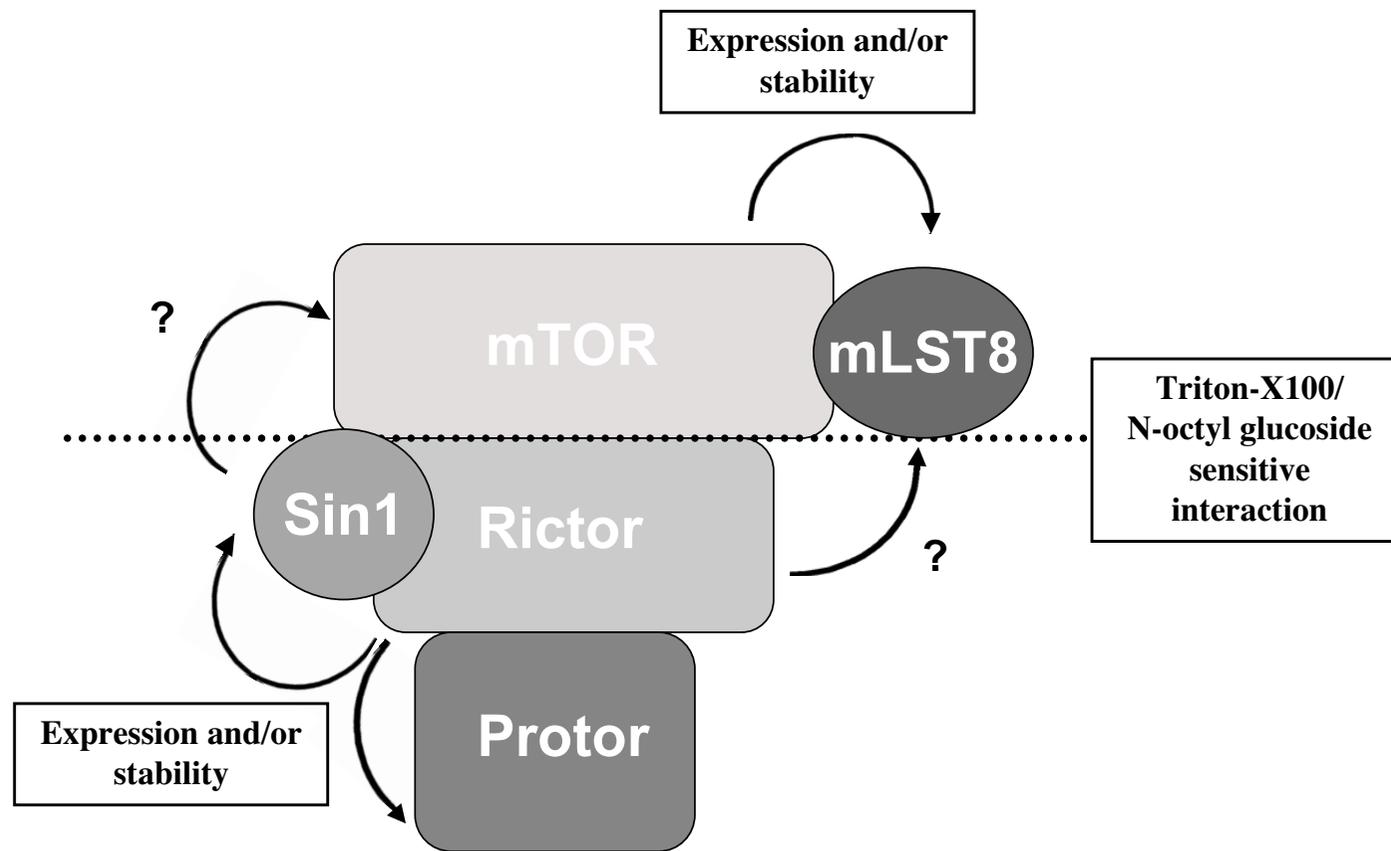


FIGURE 5