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Supplemental Data

PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase

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Supplemental Experimental Procedures

cDNA Manipulations, Mutagenesis, and Sequence Alignments

The cDNA for PRAS40 (NCBI gene symbol: AKT1S1) was obtained from Origene. The PRAS40 cDNA were amplified by PCR using pCMV6-XL5 as a template and the product subcloned into the Sal 1 and Not 1 sites of pRK5, the Xho I and Not I sites of MSCV, and the Sal I and Not 1 sites of flag-prk5. All constructs were verified by DNA sequencing. The PRAS40 cDNA in pRK5 was mutagenized using the QuikChange XLII mutagenesis kit (Stratagene) with oliogonucleotides obtained from Integrated DNA Technologies. NCBI Blosum62 Blast searches were used to identify blocks of similar sequence between PRAS40 orthologues.

Mammalian Lentiviral shRNAs

Lentiviral shRNAs to human raptor and mTOR were previously described (Sarbassov et al., 2005). All other shRNA were obtained from the collection of The RNAi Consortium (TRC) at the Broad Institute (Moffat et al., 2006). These shRNAs are named with the numbers found at the TRC public website (http://www.broad.mit.edu/genome_bio/trc/publicSearchForHairpinsForm.php):

Mouse raptor shRNA: TRCN0000077472; NM_028898.1-3729s1c1
Mouse PRAS40 #1 shRNA: TRCN0000181472; NM_026270.1-865s1c1
Mouse PRAS40 #2 shRNA: TRCC0001186119; NM_032375.2-1425
Human PRAS40 #1 shRNA: TRCC0001186123; NM_032375.2-1254
Human PRAS40 #2 shRNA: TRCN0000165801; NM_032375.2-1279s1c1

shRNA-encoding plasmids were co-transfected with the Delta VPR envelope and CMV VSV-G packaging plasmids into actively growing HEK-293T using FuGENE 6 transfection reagent as previously described (Ali and Sabatini, 2005; Sarbassov et al., 2005). Virus-containing supernatants were collected at 48 hours after transfection, filtered to eliminate cells, and target cells infected in the presence of 8 μg/ml polybrene. 24 hours later, cells were selected with puromycin and analyzed on the 2nd or 3rd day after infection.

Drosophila RNAi and Analysis

Design and Synthesis of dsRNAs

The control GFP dsRNA has been described (Sarbassov et al., 2004). Other dsRNAs were designed to target all known transcripts of the target *Drosophila* gene. In order to minimize off-target effects, we used the DRSC tool at http://flyrnai.org/RNAi_find_frag_free.html and excluded regions of 19-mer-or-greater identity to any *Drosophila* transcripts. Synthesis of dsRNAs was performed as previously described (Sarbassov et al., 2004).

Primer Sequences (Iincluding Underlined 5' and 3' T7 Promoter Sequences)

Lobe dsRNA #1 forward primer:
GAATTAATACGACTCACTATAGGGAGAGCAGGTGTTCCAGCAGAAGTAT
Lobe dsRNA #1 reverse primer:
GAATTAATACGACTCACTATAGGGAGAGTGCAGTTGATGAGGTAGAGAGTG
Lobe dsRNA #2 forward primer:
GAATTAATACGACTCACTATAGGGAGATAAATTAGTTGCTCAACGAAACAAG
Lobe dsRNA #2 reverse primer:
GAATTAATACGACTCACTATAGGGAGAAGTTAGTTAACTCAATGTGCAACAGC
dRheb dsRNA forward primer:
GAATTAATACGACTCACTATAGGGAGA AAATCGTCGCTATGCATACAGTT
dRheb dsRNA reverse primer:
GAATTAATACGACTCACTATAGGGAGAAGACTTTTGGCTGGTTATCGAGT
dTOR dsRNA forward primer:
GAATTAATACGACTCACTATAGGGAGAATTTGAAAAACGAGCTAACACAGTC
dTOR dsRNA reverse primer:
GAATTAATACGACTCACTATAGGGAGAGTACTTCAGCCAGGTATGTATGTCC
dTSC2 dsRNA forward primer:
GAATTAATACGACTCACTATAGGGAGATACCCCCTACCTTGACATTCTG
dTSC2 dsRNA reverse primer:
GAATTAATACGACTCACTATAGGGAGAATATTGCTAACGATCGAGGACATT

dsRNA Transfection and *Drosophila* Cell Culture

Kc167 were seeded in 6-well dishes at 2 X 10^6 cells per well and grown in Schneider's medium plus 10% IFS. S2 cells were seeded in 6-well dishes at 1 X 10^6 cells per well and grown in Drosophila-SFM. For single-knockdown immunoblot experiments, dsRNAs were transfected only once at 1 μg/million cells. Media was refreshed at 24 hours post-transfection and cell lysates were prepared 43 hours post-transfection. For single-knockdown cell size experiments, dsRNAs were transfected on three successive days at 1 μg/million cells, and cell sizes were measured 24 hours after the final transfection. For sequential-knockdown experiments, the first dsRNA was transfected on days 1-4 at 1 μg/million cells, and the second dsRNA was transfected on days 3-4 at 2 μg/million cells. Media was refreshed prior to transfections on day 3. Cell lysates were prepared 36 hours following the final transfection and cell sizes were measured 24 hours following the final transfection. Transfection and cell lysis conditions were previously described (Sarbassov et al., 2004).

Supplemental References

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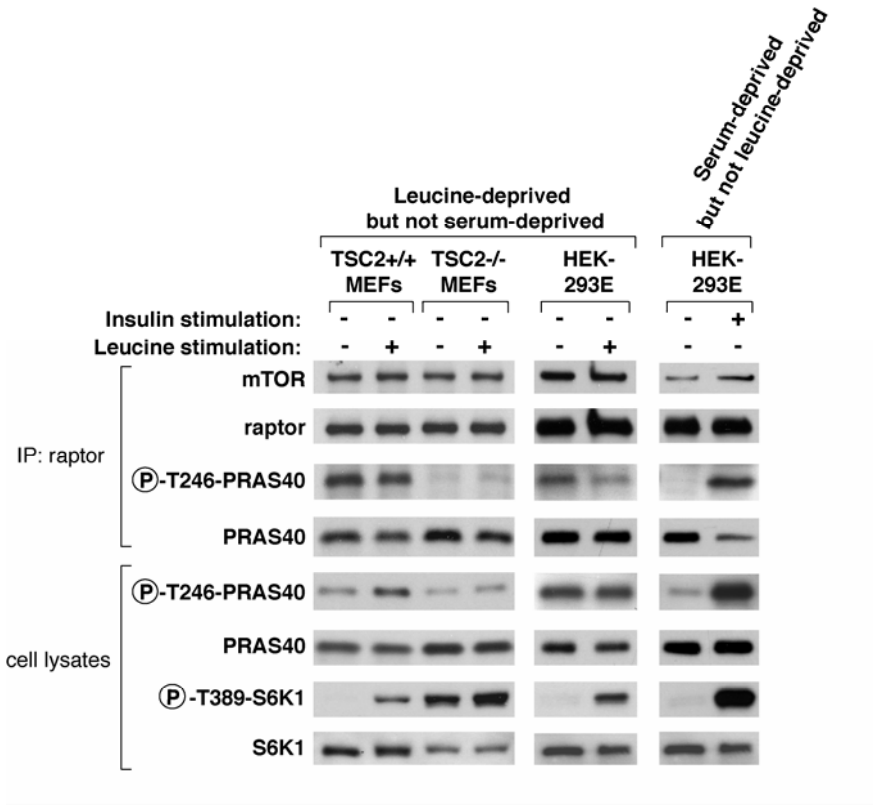

Figure S1. Leucine Has Minor Effects on PRAS Phosphorylation at T246 and the PRAS40-mTORC1 Interaction

Confluent wild-type or TSC2 null MEFs or actively growing HEK-293E cells were deprived of leucine by incubating the cells in leucine-free RPMI containing 10% dialyzed IFS. MEFs and HEK-293 cells were deprived of leucine for 5 and 1 hour(s), respectively. Cells were stimulated by adding 52 μg/ml leucine to the leucine-free serum-containing media for 15 minutes. A separate set of HEK-293E cells was starved for serum (but not leucine) for 1 hour or serum starved and then stimulated with 150 nM insulin for 15 minutes. Cell lysates and raptor immunoprecipitates were prepared and immunoblotting was used to measure the levels and phosphorylation states of the indicated proteins as described in the Experimental Procedures. Note: in contrast to our previous work (Kim et al., 2002), we do not observe a leucine-induced disruption of the raptor-mTOR interaction using our current cell lysis and purification procedures.