Published in final edited form as:
Oncogene. 2010 July 15; 29(28): 4068–4079. doi:10.1038/onc.2010.177.

Proton-assisted amino acid transporters are conserved regulators of proliferation and amino acid-dependent mTORC1 activation

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Abstract

The PI3-kinase (PI3K)/Akt and downstream mammalian target of rapamycin complex 1 (mTORC1) signalling cascades promote normal growth and are frequently hyperactivated in tumour cells. mTORC1 is also regulated by local nutrients, particularly amino acids, but the mechanisms involved are poorly understood. Unexpectedly, members of the proton-assisted amino acid transporter (PAT or SLC36) family emerged from *in vivo* genetic screens in *Drosophila* as transporters with uniquely potent effects on mTORC1-mediated growth. Here we show the two human PATs that are widely expressed in normal tissues and cancer cell lines, PAT1 and PAT4, behave similarly to fly PATs when expressed in *Drosophila*. siRNA knockdown reveals that these molecules are required for activation of mTORC1 targets and for proliferation in human MCF-7 breast cancer and HEK-293 embryonic kidney cell lines. Furthermore, activation of mTORC1 in starved HEK-293 cells stimulated by amino acids requires PAT1 and PAT4, and is elevated in PAT1-overexpressing cells. Importantly, in HEK-293 cells, PAT1 is highly concentrated in intracellular compartments, including endosomes, where mTOR shuttles upon amino acid stimulation. Our data are therefore consistent with a model in which PATs modulate mTORC1's activity not by transporting amino acids into the cell, but by modulating the intracellular response to amino acids.

Keywords

SLC36; mTORC1; PI3K; amino acid sensing; transporter; transceptor; endosome

INTRODUCTION

The Target of Rapamycin complex 1 (TORC1) signalling cascade is an evolutionarily ancient nutrient-sensitive pathway, originally identified in yeast and subsequently shown to be conserved in higher eukaryotes (Kunz *et al.*, 1993; Wullschleger *et al.*, 2006). TOR, a central component of the TORC1 complex, is a serine-threonine kinase that controls cell

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Conflict of interest statement. The authors declare no conflicts of interest

Supplementary Information accompanies the paper on the *Oncogene* website (http://www.nature.com/onc)

Europe PMC Funders Author Manuscripts

growth by regulating several molecules involved in protein translation, including p70 S6-kinase (S6K1), its ribosomal target protein S6, and the inhibitor of translational initiation, 4E-binding protein (4E-BP1; Hietakangas and Cohen, 2009). TORC1 also inhibits autophagy (Chang *et al.*, 2009). Mammalian TORC1 (mTORC1) activity is controlled by oxygen and energy levels, and by extracellular nutrients, in particular amino acids, which are taken up by cells and sensed by a currently unknown intracellular mechanism (Beugnet *et al.*, 2003; Avruch *et al.*, 2009).

Studies in *Drosophila* have revealed an important role for the PI3-kinase (PI3K)/Akt (or Protein Kinase B) signalling cassette in cell growth control (Goberdhan *et al.*, 2003). Since PI3K/Akt signalling is hyperactivated in the majority of cancers, these findings have highlighted the importance of misregulated cell growth in human tumorigenesis (Brugge *et al.*, 2007; Yuan and Cantley, 2008). Work in mammals and invertebrates has shown that PI3K/Akt signalling is critically involved in a range of other physiologically important processes, such as ageing (Tiovonen and Partridge, 2009), and its misregulation is an important factor in several other major human diseases, such as Type 2 diabetes and certain neurodegenerative disorders (Goberdhan and Wilson, 2003; Reynolds *et al.*, 2007; Kim and Mak, 2006).

Studies in flies have also been instrumental in demonstrating a link between PI3K/Akt signalling and TORC1 (Goberdhan and Wilson, 2003; Hafen, 2004). TORC1 is activated by the G-protein Rheb (Ras homologue enriched in brain). This molecule is inhibited by the GAP (GTPase-activating protein) activity of the Tuberous Sclerosis TSC1/TSC2 complex. TSC1/TSC2 can itself be blocked by activated Akt, leading to derepression of Rheb and activation of TORC1. This link highlighted the importance of mTORC1 signalling in cancer (Guertin and Sabatini, 2007), particularly in the growth regulatory control of protein synthesis. Indeed, rapamycin analogues have recently been approved by the FDA for the treatment of patients with advanced renal cell carcinoma (Hudes *et al.*, 2007; Atkins *et al.*, 2009).

The mechanisms by which mTORC1 is regulated by amino acids remain controversial (Reynolds *et al.*, 2007; Avruch *et al.*, 2009). Since tumour cells can be exposed to conditions in which growth factors, nutrients and oxygen are depleted during cancer progression (Hsu and Sabatini, 2008; Luo *et al.*, 2009), understanding how amino acids control mTORC1 and how these mechanisms might be altered in tumour cells is an important goal in cancer research, particularly since it might highlight ‘sensors’ of amino acids that could represent selective drug targets for manipulating mTORC1 signalling (Hundal and Taylor, 2009; Ganapathy *et al.*, 2009).

Studies in cell culture have suggested a role for several amino acid transporters (AATs), including the solute-linked carrier (SLC)1A5 glutamine transporter (otherwise known as ASCT2; alanine-, serine-, cysteine- preferring transporter; Fuchs and Bode, 2005; Nicklin *et al.*, 2009), the heterodimeric CD98 (SLC7A5/SLC3A2) bidirectional amino acid exchanger (Nicklin *et al.*, 2009), and the sodium-coupled neutral amino acid transporter 2 (SNAT2, a member of the SLC38 family [Hundal and Taylor, 2009]) in amino acid-dependent mTORC1 activation. In those cases where the mechanism has been characterised, these transporters are involved in bringing essential amino acids that activate mTORC1, in particular leucine, into the cell (e.g., Nicklin *et al.*, 2009). A number of genes have also been implicated in the process of intracellular nutrient sensing (reviewed in Avruch *et al.*, 2009), e.g., MAP4K3 (Findlay *et al.*, 2007), Vps34 (Byfield *et al.*, 2005; Nobukuni *et al.*, 2005) and the RAG GTPases (Sancak *et al.*, 2008; Kim *et al.*, 2008). Studies of RAG function led to the proposal that mTORC1 can be activated by amino acids through shuttling to late endosomal compartments (Sancak *et al.*, 2008). However, the precise mechanisms involved

and the intracellular amino acid sensor remain undiscovered. Furthermore, to date, only the RAGs have been shown to specifically modulate mTORC1 signalling *in vivo*, using genetic approaches in *Drosophila* (Kim *et al.*, 2008).

We used an alternative *in vivo* approach to dissect the role of AATs in TORC1 regulation, testing the growth effects of overexpressing and mutating AATs, under otherwise normal physiological conditions in flies. This screen identified a completely different set of AATs, the proton-assisted amino acid transporter (PAT or SLC36) family, as being uniquely potent transporters in the cell-autonomous regulation of growth and TORC1 signalling (Goberdhan *et al.*, 2005). The PATs were previously identified in mammals as proton-coupled AATs located on the surface of lysosomes (Sagné *et al.*, 2001) and endosomes (Rubio-Alliaga *et al.*, 2004), as well as on the plasma membrane (Boll *et al.*, 2002; Chen *et al.*, 2003), suggesting that unlike other putative mTORC1-regulatory AATs, they could act via an intracellular mechanism. However, until now, it has not been clear whether mammalian PATs can promote growth.

Here we show that the two ubiquitously expressed human PATs, PAT1 and PAT4, have growth regulatory activity when expressed in flies and display genetic interactions consistent with a role in mTORC1 signalling. Using a siRNA knockdown strategy, we find that reducing the levels of these molecules strongly inhibits cell proliferation and mTORC1 signalling in human MCF-7 breast cancer cells and HEK-293 embryonic kidney cells. Remarkably, PAT1 and PAT4 are required in starved HEK-293 cells for the normal rapid response of mTORC1 to amino acid addition, even though PAT1 is located intracellularly. Furthermore, overexpression of PAT1 can stimulate this response. We conclude that PATs are important conserved regulators of amino acid-dependent TORC1 activation and propose that they may perform this function by participating in intracellular amino acid sensing.

RESULTS

PAT1 and *PAT4* are highly expressed in a wide range of human cancer cell lines and normal tissues

In contrast to the eleven PATs predicted in the fly genome, there are only four PATs in mammals. The tissue distribution of these molecules and their mRNAs has been characterised in a number of species (Bermingham and Pennington, 2004). These studies, which include quantitative-RT-PCR (Q-RT-PCR) and northern blot analysis in humans (Nishimura and Naito, 2005), have indicated that while *PAT1* and *PAT4* are both expressed in most or all tissues, the other human PATs, *PAT2* and *PAT3*, are expressed in a limited range of cell types.

We analysed *PAT* mRNA levels in a number of human cancer cell lines derived from different tissues using Q-RT-PCR, comparing expression levels to those of the abundantly expressed *hypoxanthine-guanine phosphoribosyl transferase* (*HPRT1*) housekeeping gene (Table 1). Consistent with studies in normal human tissues, *PAT1* and *PAT4* are expressed at moderate to high levels in all lines tested (5% to 130% of *HPRT1* levels). There was no detectable expression of *PAT2* or *PAT3* (data not shown). Different cell lines derived from the same tissue showed up to ten fold differences in *PAT1* and *PAT4* expression (e.g., *PAT4* in HCT116 and DLD1 colon cancer lines). cDNAs derived from several normal tissues revealed expression levels broadly comparable to cancer cell lines from the same tissue. Based on their ubiquitous expression, we concluded that PAT1 and PAT4 are the most likely PAT transporters to be involved in global regulation of growth and proliferation.

Human *PAT1* and *PAT4* have similar properties to *Drosophila* PATs when expressed in flies

Overexpression of genes in post-mitotic cells of the developing fly eye using the GAL4/UAS system (Brand and Perrimon, 1993) provides a sensitive means of screening for genes that regulate growth, and revealed that the *Drosophila* PATs are growth activators (Goberdhan *et al.*, 2005). To determine whether human PATs can promote growth *in vivo*, we generated transgenic fly lines in which human *PAT1* or *PAT4* gene expression can be controlled by GAL4. Expression of *PAT1* or *PAT4* in the differentiating fly eye under the control of the GMR-GAL4 driver induced modest levels of overgrowth similar to the fly PAT transporter *path* (Figure 1A-D). We previously found that co-expression in the differentiating eye of fly PATs or other growth-promoting TORC1-activating proteins, such as Rheb and S6K1, with the transcription factor FOXO, enhances the characteristic FOXO-induced apoptotic phenotype, an effect not observed with other TORC1-independent growth regulators (Goberdhan *et al.*, 2005). In flies, TORC1 signalling interferes with the activation of Akt, a key negative regulator of FOXO, via a negative feedback mechanism (Radimerski *et al.*, 2002a; Radimerski *et al.*, 2002b), providing an explanation for this characteristic PAT/FOXO genetic interaction. Co-expression of *PAT1* or *PAT4* with *foxo* enhanced the FOXO-induced reduced eye phenotype in a similar manner to *path* (Figure 1E-H). Both *Drosophila* and human PATs therefore promote growth and genetically act like TORC1 regulators *in vivo*, at least in the fly system.

PAT1 and PAT4 are essential for proliferation in MCF-7 breast cancer cells

The roles of PAT1 and PAT4 in the regulation of tumour cell proliferation were tested in the human MCF-7 breast cancer cell line. The PI3K/Akt signalling cassette is hyperactivated in these cells, because they carry an activating mutation in the PI3K family member PI3KCA (Hollestelle *et al.*, 2007).

PAT1 and *PAT4* mRNAs were knocked down using three independent siRNAs for each transcript to control for off-target effects. Cells were cultured for 120 h and their proliferation was assessed by counting the cells blind in a randomised fashion. Counts for *PAT* knockdowns were compared to cells that were untreated, treated with transfectant only or transfected with a scrambled siRNA sequence. In further experiments, three independent *mTOR* siRNAs were used to test the effects of reducing mTOR-dependent signalling in these cells. Q-RT-PCR revealed that knockdown of *PAT1*, *PAT4* and *mTOR* produced 60-90% reductions in levels of the targeted transcripts and that this was achieved within 24 h of transfection (Supplementary Figure 1).

mTOR, *PAT1* and *PAT4* siRNAs all had similar effects on cell proliferation in these experiments (Figure 2). Compared to controls, cell amplification over the 120 h time-course was reduced by more than 75%. All knockdown samples contained similar numbers of dead cells compared to controls, as assessed by trypan blue staining (generally less than 10% of cells were non-viable; Supplementary Table 1), suggesting that the changes in cell number were not primarily due to elevated cell death. Furthermore, there was no significant increase in the total numbers of detached and dead cells in the culture medium (Supplementary Table 1). There were also no obvious differences in the morphology of the cells (data not shown). Simultaneous knockdown of both *PAT1* and *PAT4* using the most effective siRNAs for each of these genes, si159 and si437, had no significant additional effect on MCF-7 cell number. We conclude that both PAT1 and PAT4 are required to promote proliferation of MCF-7 cells under normal culture conditions.

PAT1 and PAT4 modulate mTORC1 signalling in MCF-7 breast cancer cells

To test whether PAT1 and PAT4 modulate mTORC1 activity in human cells, protein extracts from knockdown MCF-7 cells were analysed by western blotting using antisera raised against phosphorylated forms of multiple mTORC1 signalling targets, namely Phospho-Thr-389-S6K1, Phospho-Ser-240/244-S6 and PhosphoSer-65-4E-BP1. As expected, *mTOR* knockdown strongly reduced the levels of phosphorylated S6K1 and 4E-BP1 compared to controls (Figure 3A and 3B). Both *PAT1* and *PAT4* knockdown cells also showed reduced phosphorylation of these mTORC1 targets. By contrast, these treatments did not alter PI3K/Akt signalling, as judged by staining with anti-Phospho-FoxO1/FoxO3a antiserum. Therefore, the inhibition of proliferation caused by decreased expression of PAT transporters is presumably at least partly explained by a reduction in mTORC1 signalling. Coupled with our *in vivo* data in flies, these experiments demonstrate that PAT transporters have an evolutionarily conserved role in regulating mTORC1 signalling.

PAT1 and PAT4 modulate the response of mTORC1 to amino acids in human embryonic kidney HEK-293 cells

The identification of PATs as regulators of mTORC1 signalling was initially unexpected, because mammalian PATs frequently localise not at the cell surface, but in endosomal and lysosomal compartments within cells (Sagné *et al.*, 2001; Rubio-Alliaga *et al.*, 2004; Wreden *et al.*, 2003). However, Sancak *et al.* (2008) have found that mTOR is translocated to late endosomes upon amino acid stimulation in HEK-293 cells, suggesting that mTORC1 activation may involve an amino acid-dependent event in this compartment. Staining of MCF-7 and HEK-293 cells with a rabbit antiserum against PAT1 (Wreden *et al.*, 2003) revealed that PAT1 is located intracellularly within vesicular structures that are concentrated around the nucleus (Figures 4A and 4B respectively). HEK-293 cells stably overexpressing PAT1 also showed a similar, but more intense, perinuclear vesicular pattern, confirming the specificity of the antibody staining (Figure 4C). Although most of the antibodies previously used to study subcellular localisation of mTORC1 signalling in these cells were also generated in rabbit (Sancak *et al.*, 2008), we employed a co-transfected Rheb-GFP construct (Buerger *et al.*, 2006), which marks mTORC1-activating Rheb and is located in the cytoplasm around late endosomes, primarily at one side of the nucleus in HEK-293 cells (Sancak *et al.*, 2008). It frequently localised directly adjacent to or surrounded PAT1 protein (Figures 4D and 4E), indicating that as in other cell types, PAT1 is at least partly localised to endosomal structures.

Knockdown of *mTOR*, *PAT1* and *PAT4* in HEK-293 cells significantly reduced cell number after 72 h of culture (Figure 4F-I). We used a different more transient transfection protocol for knockdown in these experiments to facilitate subsequent analysis. As a result, *PAT* transcript levels were less strongly decreased than in MCF-7 cells (compare Supplementary Figures 1 and 2), but western analysis with anti-mTOR and anti-PAT1 antibodies confirmed that protein levels were strongly reduced by the knockdown procedure (Supplementary Figure 2D and 2E). Again there was no evidence for increased levels of dead cells in knockdown samples (Supplementary Table 2) and less than 1% of cells detached from the culture well, indicating that the reduction in cell number caused by either *PAT* or *mTOR* knockdown was primarily caused by decreased proliferation. Although we did not specifically study the effect of PAT and mTOR depletion on growth, total protein and RNA levels extracted from HEK-293 knockdown cells were typically reduced relative to controls (Supplementary Table 3), suggesting that net growth of these cells is also inhibited.

mTOR, *PAT1* and *PAT4* knockdown all significantly reduced levels of phosphorylated S6K1, S6 and 4E-BP1, unlike the scrambled siRNA and MATra-treated controls (Figure 5), but had a much more subtle effect on PI3K/Akt signalling, as judged by staining with anti-

Phospho-FoxO1/FoxO3a antiserum. Phosphorylation of Akt at Ser-473, which is mediated by the mTOR-containing mTORC2 complex, was unaffected, indicating that mTORC2 and PI3K/Akt signalling is not strongly modulated by mTOR or PAT knockdown under these conditions.

To test whether PATs are specifically involved in modulating mTORC1's sensitivity to amino acids, we starved normal and knockdown cells of serum and nutrients for 50 min after 72 h of culture, then added back amino acids for 30 min, before assessing phosphorylation of mTORC1 targets (Sancak *et al.*, 2008). Knockdown of *PAT1* and *PAT4*, as well as *mTOR*, strongly reduced amino acid-dependent mTORC1 signalling (Figure 6A-D), but not mTORC2 signalling, as judged by Akt phosphorylation. In addition, a stable cell line overexpressing PAT1 (Figure 4C), which proliferates more rapidly than cells transfected with empty vector (Figure 6F), showed an enhanced response to amino acids without affecting basal signalling in amino acid-starved cells (Figure 6E), strongly suggesting that PATs do not merely play a permissive role in this process. We conclude that PATs are important intracellular regulators of TORC1, which modulate the sensitivity of this complex to amino acids.

DISCUSSION

We have previously identified the PAT amino acid transporters as uniquely potent AATs in the *in vivo* regulation of TORC1-mediated growth in *Drosophila*. Here we demonstrate for the first time that two human PATs, PAT1 and PAT4, have similar *in vivo* activities in flies and are also required for proliferation in the human breast cancer cell line MCF-7 and embryonic kidney cell line HEK-293. Furthermore, we demonstrate that these PATs are involved in the regulation of mTORC1 signalling and that they are required for amino acid-dependent activation of this cascade in HEK-293 cells. Since PAT1 resides in intracellular compartments within these cells rather than at the cell surface, we propose that the PATs are likely to act via a novel aspect of amino acid sensing that does not involve simple transport of nutrients across the plasma membrane.

Knockdown of *PAT1* or *PAT4* produces non-redundant inhibition of cell proliferation and mTORC1 signalling

The presence of four mammalian PATs versus eleven predicted PATs in the fly facilitated genetic analysis in human cells. Knockdown of *PAT1* or *PAT4* produced a strong inhibition of proliferation in MCF-7 and HEK-293 cell lines, and in HEK-293 cells, we also demonstrated a clear reduction in total RNA and protein, suggesting that growth is also affected. Multiple siRNAs for each gene produced 55-90% reductions in mRNA levels and typically more than a 60% decrease in cell amplification. Cell survival was not significantly affected, suggesting that blocking PAT activity is cytostatic rather than cytotoxic. Knockdown of *mTOR* produced similar effects on cell number without affecting the number of dying cells.

Several lines of evidence indicate that the pro-proliferative properties of PAT1 and PAT4 are at least partly mediated through mTORC1. First, these molecules behave similarly to the *Drosophila* PATs and also other molecules in the mTORC1 signalling cascade in a FOXO-dependent genetic interaction screen in flies (Goberdhan *et al.*, 2005). Second, in MCF-7 and HEK-293 cells, knockdown of either *PAT1* or *PAT4* reduces the phosphorylation of the mTORC1 targets S6K1, S6 and 4E-BP1, but does not affect mTORC2 or PI3K/Akt signalling. Third, PATs are important in determining the sensitivity of mTORC1 to amino acids under growth factor-deficient conditions, highlighting a specific role for these transporters in this process. Furthermore, elevated expression of PAT1 increases the

sensitivity of mTORC1 to amino acids, strongly supporting a modulatory role for PAT1 in mTORC1's response.

We were surprised to find that both *PAT1* and *PAT4* knockdown had similar effects on proliferation, and that reducing levels of both genes simultaneously in MCF-7 cells had no further inhibitory action. One possible explanation for the lack of redundancy is that PAT1 and PAT4 act together to regulate mTORC1 activity. Indeed, the functional forms of several other amino acid and peptide transporters appear to be multimeric, for example CD98 (Devés and Boyd, 2000) and PepT1 (Panitsas *et al.*, 2006). Alternatively, PAT1 and PAT4 may have distinct roles in mTORC1 control.

Regulation of mTORC1 signalling by PAT transporters

Studies in cell culture have highlighted several cell surface AATs as putative mTORC1 activators (Hundal and Taylor, 2009; Ganapathy *et al.*, 2009; Nicklin *et al.*, 2009). A critical observation that has shaped this work is that, intracellular leucine and other essential amino acids (EAAs) can act as potent activators of mTORC1 (Beugnet *et al.*, 2003) and that in *Xenopus* oocytes changes in intracellular leucine alter mTORC1 activity (Christie *et al.*, 2002). These mTORC1-regulatory transporter systems are, therefore, thought to work by modulating intracellular leucine and EAA levels through transport across the plasma membrane (Nicklin *et al.*, 2009). However, the downstream leucine/EAA sensor remains to be identified.

In our *in vivo* genetic screen for AATs that regulate growth and TOR signalling (Goberdhan *et al.*, 2005), we had expected to identify other cell surface AATs that bring amino acids into the cell. However, several lines of evidence suggest that this is not the primary role of the PATs. First, in HEK-293 cells, where we have shown a clear role for PATs in amino acid-dependent stimulation of mTORC1, PAT1 is located intracellularly (Figure 4B). PAT1 is also found in the endosomes of HeLa cells (Wreden *et al.*, 2003), which we have found require PATs for normal proliferation (data not shown), and in MCF-7 cells (Figure 4A), suggesting that this may be a common subcellular distribution in rapidly dividing cells in culture. Second, the proton-assisted mechanism employed by PAT transporters is consistent with a role in endosomal and lysosomal, not cell surface, transport (Boll *et al.*, 2004). Third, there is currently no evidence that PAT1 acts as a transporter for leucine or mTORC1-activating EAAs with alanine, proline and histidine being its characterised amino acid substrates (Boll *et al.*, 2004). However, since PAT4 remains an orphan transporter of unknown subcellular localisation, we cannot eliminate the possibility that this molecule does have a cell surface function in mTORC1 regulation.

Our data are more consistent with a model in which intracellular PATs alter the sensitivity of mTORC1 to amino acids. Recent studies in HEK-293 cells have shown that in the presence of amino acids, mTOR is relocated to late endosomal compartments around which Rheb accumulates (Sancak *et al.*, 2008), suggesting that some aspects of mTORC1's amino acid-sensing mechanism involve endosomal signalling (Cohen and Hall, 2009). Although PATs could have a role in any aspect of transmitting the amino acid signal from the cortical cytoplasm to mTORC1, the endosomal location of these transporters in many cell types and their abilities to interact directly with amino acids lead us to favour a model in which PATs participate in the intracellular amino acid sensing mechanism (Figure 7). PATs may interact either directly or indirectly with endosomal mTORC1 to control the response to amino acids. This activity could involve amino acid transport from the endosomal lumen or a signalling mechanism, since even PATs with low amino acid transport capacity can promote growth in flies, leading to the suggestion that they can act as so-called transceptors (Goberdhan *et al.*, 2005). Since the activity of transceptors can be affected by the concentration of substrate-like molecules on their outer (in this case cytoplasmic) face

(Hundal and Taylor, 2009), PATs could act directly as sensors for cytoplasmic leucine and EAAs (Figure 7). Alternatively, they may merely provide an input to another molecule that acts as the amino acid sensor for mTORC1 and is responsive to PAT activity and cytoplasmic leucine/EAAs. If the PATs are direct sensors of amino acids, this would provide an important functional link between these transporters and cell surface amino acid transporters that modulate mTORC1 activity by altering cytoplasmic amino acid levels (Zinzalla and Hall, 2008; Goberdhan *et al.*, 2009). The recent identification of the Rags as key mediators of amino acid-dependent mTORC1 activation (Sancak *et al.*, 2008) is of particular interest in this regard, because the orthologues of these molecules in yeast have been implicated in shuttling amino acid transporters to their appropriate subcellular compartment (Gao and Kaiser, 2006).

It will now be important to develop tools to establish the precise localisation of PATs in the cell, to determine whether they modulate mTORC1 activity by direct or indirect interaction with mTORC1 signalling components, and to establish how they are shuttled around the cell. Ultimately, understanding the mechanism of PAT action may suggest new strategies to modulate mTORC1 activity in cancer and other human diseases where this pathway is misregulated.

MATERIALS AND METHODS

Cell culture and siRNA transfection conditions for MCF-7 cells

MCF-7 cells were typically cultured in 24-well plates (well diameter 1.88 cm^2) at seeding densities of 2.5×10^5 per well in medium containing 10% foetal bovine serum (FBS; Gibco™ Invitrogen). They were transfected 24 h after plating, when they were approximately 30% confluent. Transfections were performed using lipofectamine™ RNAimax (Invitrogen) following the manufacturer's instructions with all dilutions carried out in serum-reduced optiMEM® medium (Invitrogen). Cells were incubated with the siRNA complex over a time-course of 120 h.

Cell culture and siRNA transfection conditions for HEK-293 cells

HEK-293 cells were cultured in 6-well, 12-well or 24-well plates at seeding densities of 3.0×10^5, 1.2×10^5 or 0.6×10^5 cells per well respectively, in MEM (Minimum Essential Medium Eagle; Sigma-Aldrich) containing 10% FBS (Gibco™ Invitrogen), 1 × MEM-NEAA (Sigma-Aldrich), and 4 mM L-Glutamine (Sigma-Aldrich). They were transfected 24 h after plating, when they were approximately 40% confluent. Transfections were performed using the MATraA magnet-assisted transfection protocol (IBA Technologies) following the manufacturer's instructions with all dilutions carried out in serum-reduced optiMEM® medium (Invitrogen). Cells were incubated with the siRNA complex over a time-course of 72 h.

Cell counts

For cell counts from each well, cells in the medium and attached cells were separately harvested and then counted in triplicate using a haemocytometer (improved Neubauer model 2007 [Sigma]) in a randomised, blind manner. Cell viability was measured using trypan blue. In the case of MCF-7 cells, the remaining cells from the triplicate wells were pooled and pelleted by centrifugation for protein analysis. HEK-293 lysates were collected from separate wells.

Amino acid starvation

Three days after transfection, normal medium was removed from the culture plates. Cells were rinsed once with amino acid-free RPMI-1640 medium (US Biological). Cells were

starved for 50 min in the amino acid-free medium (at 37°C, 5% CO_2) and then stimulated with amino acids for 30 min. A 50 × RPMI amino acid solution (Sigma-Aldrich) was added to the starvation medium to give a final amino acid concentration equivalent to RPMI-1640 media. After 30 min, medium was discarded, the cells were washed twice with ice-cold PBS and then lysed in RIPA buffer containing protease and phosphatase inhibitor (Sigma-Aldrich) for 30 min. Lysates were cleared by centrifugation (13 000 rpm, 10 min in a microcentrifuge at 4°C) and either stored at −20°C or used immediately.

Generation of stable cell line

HEK-293 cells were transfected with a pcDNA™3.1(+) (Invitrogen) construct containing PAT1 under the control of the CMV promoter or the empty pcDNA™3.1(+) vector using FuGene®6 (Roche) as per manufacturer's instructions. Two days after transfection the cells were split into the selection medium (MEM, 10% FBS, 1 × MEM-NEAA, 4 mM L-Glutamine) which contained 800 μg/ml Geneticin® (GIBCO™ Invitrogen). The Geneticin® concentration (800 μg/ml) used for selection was determined by serial dilution. Medium was replaced every two to three days. After 10-15 days single colonies were picked and expanded in 24-well plates. Expression of the transgene was monitored by immunostaining and western blot analysis.

Statistical Analysis

Each experiment was performed at least three times unless otherwise stated, producing similar results. Statistical significance was determined by an unpaired one-tailed or two-tailed Student's t-test with $P<0.05$ considered as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Toshiro Aigaki, Hugo Bellen and the Bloomington Stock Centre for fly strains, Robert Edwards (UCSF) for generously providing the PAT1 antisera and Vuk Stambolic (Toronto) for the GFP-Rheb construct. We are grateful to Dr David Meredith (Oxford Brookes University) for helpful discussion and to Bass Hassan and Claudia Buehnemann for comments on the manuscript. This work was supported by Cancer Research-UK grants (C19591/A9093, C191591/A6181 and C7713/A6174), funding from the Studienstiftung des deutschen Volkes and the Max Weber Programm Bayern to SH and a Newton Abraham Studentship and Overseas Research Student Award to MHO.

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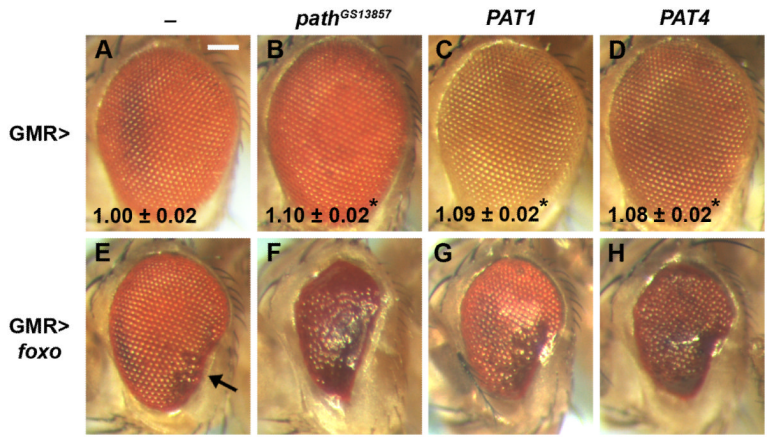

Figure 1.

Human PAT1 and PAT4 have similar activities to *Drosophila* PATs in flies. GMR-GAL4 was used to overexpress the following UAS-coupled transgenes in the differentiating cells of the eye: the fly PAT *path* from a transposable element insertion in the gene (*path*^{GS13857}; B), human *PAT1* (C) and human *PAT4* (D). All produced modest, but significant, increases in ommatidial size relative to non-expressing controls (A). The ommatidial loss induced by overexpressing *foxo* from a transposable element insertion in the gene, *foxo*^{GS9928}, using *GMR-GAL4* is most clearly seen at the ventro-posterior edge of the eye (arrow in E). It is enhanced by co-overexpression of both fly and human PAT transporters (F-H). Scale bar is 100 μm and applies to all panels. Ommatidial size measurements shown at bottom of panels A-D, mean ± s.d. relative to control; * P<0.001, n=6.

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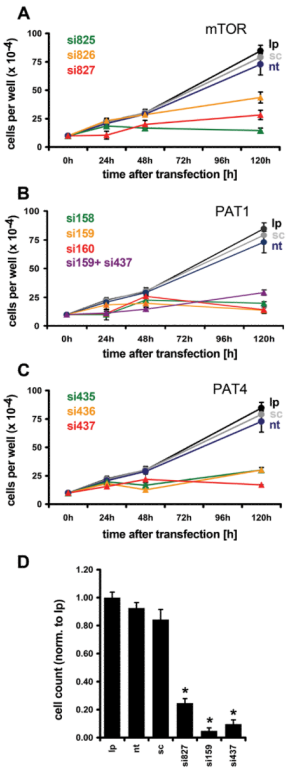

Figure 2.

PAT1 and PAT4 are both required for normal proliferation of MCF-7 breast cancer cells. The proliferation of MCF-7 breast cancer cells after transfection with siRNAs for *mTOR* (si825, si826 and si827; A), *PAT1* (si158, si159 and si160; B) and *PAT4* (si435, si436 and si437; C) was compared to control cells treated with a scrambled siRNA control (sc), lipofectamine™ only (lp) and non-transfected (nt). A combination of the most effective *PAT1* and *PAT4* siRNAs did not reduce cell number further (B). Bar chart (D) shows cell number at 120 h for samples treated with the most effective siRNAs for *mTOR* (si827), *PAT1* (si159) and *PAT4* (si437) normalised to lp control. Error bars represent SEM. *P<0.0002: reduced versus lp control. All measurements were made on triplicate samples and the experiment was repeated three times with similar results.

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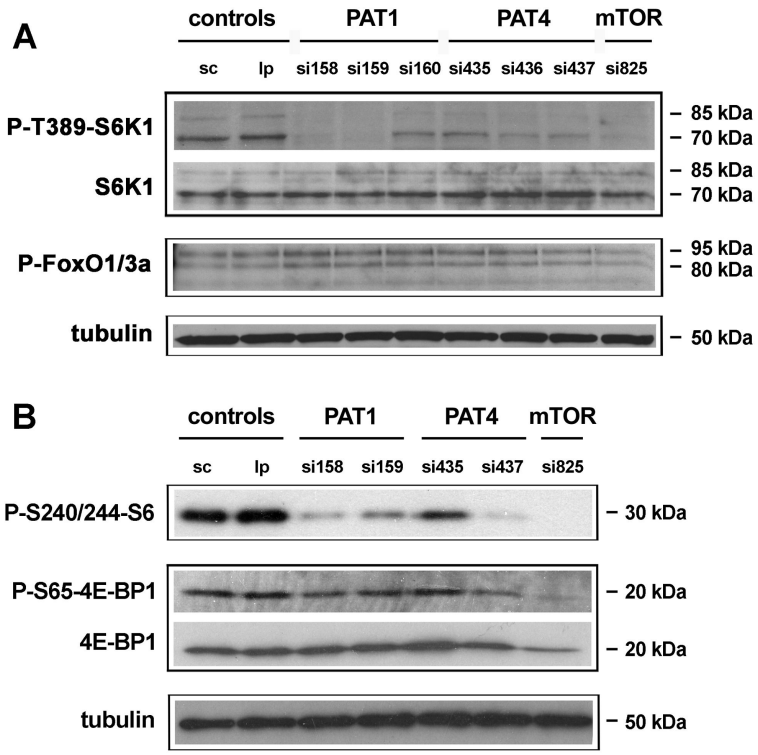

Figure 3.

PAT1 and PAT4 modulate the activity of the mTORC1 signalling cascade in MCF-7 breast cancer cells. Lysates from MCF-7 cells grown in medium containing 10% serum treated for 120 h with siRNA against *PAT1* (si158, si159 and si160), *PAT4* (si435, si436 and si437) and *mTOR* (si825) were compared to control lysates from cells treated with a scrambled siRNA (sc) or lipofectamine™ only (lp) by probing with anti-Phospho-T389-p70 S6K1 (P-T389-S6K1) and anti-Phospho-T24-FoxO1/T32-FoxO3a (P-FoxO1/3a; A), anti-Phospho-S240/244-S6 (P-S240/244-S6) and anti-Phospho-S65-4E-BP1 (P-S65-4E-BP1; B), as well as non-Phospho-specific antisera against S6K1 and 4E-BP1, and an anti-α-tubulin antibody to confirm equal loading. All experiments were repeated at least three times with similar results.

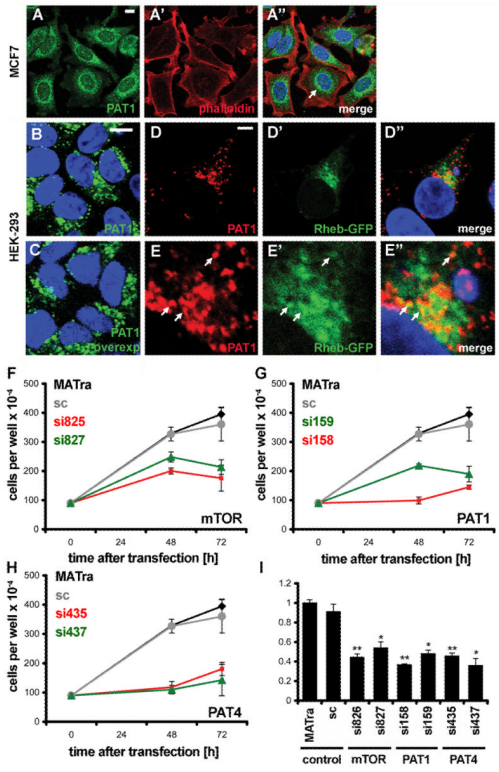

Figure 4.

PAT1 is located inside MCF-7 and HEK-293 cells from where it regulates cell proliferation. An antiserum against human PAT1 reveals a cytoplasmic localisation of endogenous PAT1 protein in both MCF-7 (A) and HEK-293 (B) cells. HEK-293 cells overexpressing PAT1 produce a much stronger signal with this antibody (C; the detection gain is reduced compared to B in this confocal image), but the protein distribution is very similar to the endogenous protein pattern. PAT1-containing structures are frequently surrounded by (top arrow), overlapping (lower arrows) or adjacent to regions containing a GFP-Rheb fusion protein, which was previously shown to reside around late endosomal compartments in HEK-293 cells (see low and high magnification views in D and E respectively). Other PAT1-containing intracellular structures may represent early endosomal or lysosomal compartments, where PATs are located in other cell types. The proliferation of HEK-293 human embryonic kidney cells after transfection with siRNAs for *mTOR* (si825 and si827; F), *PAT1* (si158 and si159; G) and *PAT4* (si435 and si437; H) was compared to control cells treated with a scrambled siRNA (sc), or with the MATra reagent only (MATra). Bar chart (I) shows cell number at 72 h for samples treated with each siRNA normalised to sc control. Error bars represent SEM. *P<0.01; **P<0.001; reduced versus MATra control. All cell number measurements were made on triplicate samples and the experiment was repeated three times with similar results.

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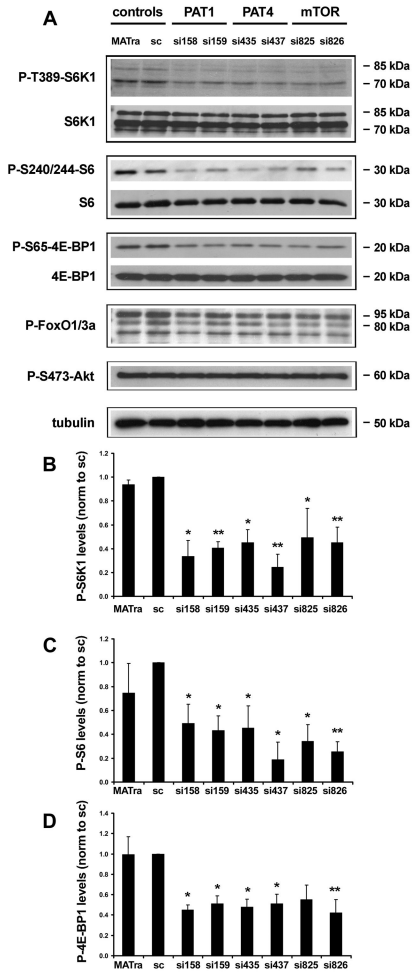

Figure 5.

PAT1 and PAT4 are required for normal mTORC1 signalling in HEK-293 cells. A. Lysates from HEK-293 cells grown in medium containing 10% serum for 72 h after transfection with siRNAs against *PAT1* (si158 and si159), *PAT4* (si435 and si437) and *mTOR* (si825 and si826) were compared to control lysates from cells treated with a scrambled siRNA (sc) or with the magnet-assisted transfection protocol without siRNA (MATra). Blots were probed with anti-Phospho-Thr-389-p70 S6K1 (P-T389-S6K1), anti-Phospho-Ser-240/244-S6 (P-S240/244-S6), anti-Phospho-S65-4E-BP1 (P-S65-4E-BP1), anti-Phospho-T24-FoxO1/T32-FoxO3a (P-FoxO1/3a), anti-Phospho-S473-Akt (P-S473-Akt) antibodies, non-Phospho-specific antisera against S6K1, S6 and 4E-BP1, and an anti-α-tubulin antibody to confirm equal loading. All experiments were repeated at least three times with similar results. Intensity measurements made from blots produced in three independent experiments reveal significant reduction in S6K1 (B), S6 (C) and 4E-BP1 (D) activation in response to *PAT1*, *PAT4* and *mTOR* knockdown. *P<0.5; **P<0.001.

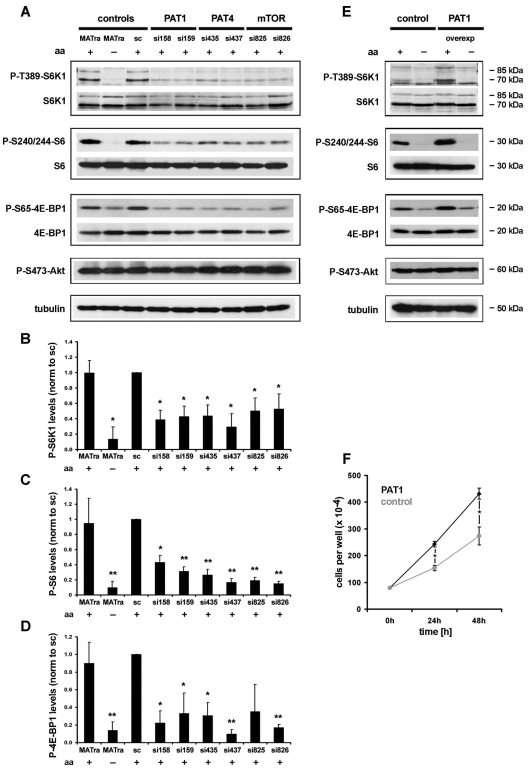

Figure 6.

PAT1 and PAT4 are required for normal activation of the mTORC1 signalling cascade by amino acids in starved HEK-293 cells. A. HEK-293 cells were grown in medium containing 10% serum for 72 h after transfection with siRNA against *PAT1* (si158 and si159), *PAT4* (si435 and si437), *mTOR* (si825 and si826), a scrambled siRNA (sc) or after exposure to MATraA without siRNA (MATra). They were starved of serum and amino acids for 50 min and then exposed to amino acids for 30 min. Extracts from these cells and cells that were not exposed to amino acids after starvation were analysed by western blot probed with anti-Phospho-Thr-389-p70 S6K1 (P-T389-S6K1), anti-Phospho-Ser-240/244-S6 (P-S240/244-S6), anti-Phospho-Ser-65-4E-BP1 (P-S65-4E-BP1), anti-Phospho-Ser-473-Akt (P-S473-Akt) antibodies, non-Phospho-specific antisera against S6K1, S6 and 4E-BP1, and an anti-α-tubulin antibody to confirm equal loading. All experiments were repeated at least three times with similar results. Intensity measurements made from blots produced in three independent experiments reveal significant reduction in S6K1 (B), S6 (C) and 4E-BP1 (D) activation in response to *PAT1*, *PAT4* and *mTOR* knockdown. *P<0.05; **P<0.001. (E) HEK-293 cells stably transfected with empty vector and a PAT1 overexpression vector were grown in medium containing 10% serum for 72 h, starved of serum and amino acids. Cultures were then exposed either to medium containing amino acids or to starvation medium for a further 30 min. Extracts from these cells were analysed by western blot with the antibodies described above. All experiments were repeated at least three times with similar results. (F) The proliferation of PAT1-overexpressing cells was compared to non-overexpressing cells over a 48 h time course (n=6). *P<0.001, proliferation significantly greater than non-expressing cells.

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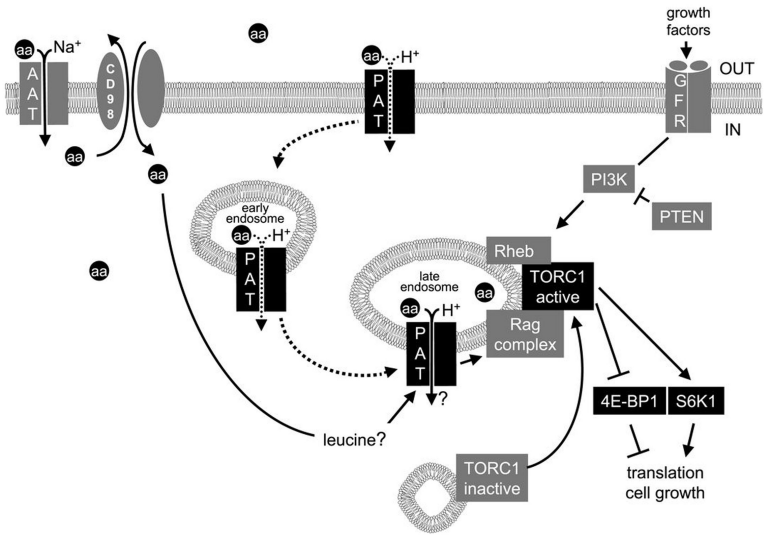

Figure 7.

A model to explain the effects of the PATs on mTORC1 signalling and proliferation. We have shown that PATs modulate the response of mTORC1 to extracellular amino acids in HEK-293 cells, even though one of the critical PATs in this process, PAT1, is concentrated intracellularly. We propose that these intracellular PATs are likely to affect mTORC1 signalling in late endosomes to which mTOR is shuttled upon amino acid stimulation. They may act via a transport-dependent or transceptor mechanism, forming a complex with other mTOR regulatory proteins, or they may be components of one of a series of endosomal complexes involved in this process. Cytoplasmic leucine, which activates S6K1, may bind to the cytoplasmic face of the PATs to enhance their ability to activate mTORC1. Rag GTPases are required to shuttle mTOR to Rheb-containing endosomal compartments upon amino acid stimulation, but evidence in yeast suggests they may also bind to amino acid transporters and promote their shuttling. Rag-dependent shuttling of PATs to the endosomes may therefore be a critical aspect of mTOR regulation.

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Table 1

PAT1 and *PAT4* are expressed in a wide range of human cancer cell lines. Expression levels of *PAT1* and *PAT4* were measured in multiple cancer cell lines (shaded grey) and in cDNA samples from normal tissue by Q-RT-PCR. Values are expressed as a percentage of the level of the ubiquitously expressed *HPRT1* housekeeping gene transcript within each cell line as measured by Ct value.

Cell Line/cDNA	Tissue	PAT1	PAT4
AsPC- I	pancreas	9%	40%
HeLa	cervix	7%	34%
DU145	prostate	11%	51%
LnCAP	prostate	11%	28%
PC3	prostate	24%	130%
NCI-H460	lung	14%	25%
A549	lung	5%	62%
WI38	lung	67%	83%
MCF-7	breast	14%	21%
MDA468	breast	9%	26%
HepG2	liver	35%	56%
Caco2	colon	55%	99%
HCT116	colon	6%	63%
DLD1	colon	10%	9%
cDNA	pancreas	10%	8%
cDNA	lung	28%	54%
cDNA	breast	32%	24%