The EMBO Journal vol.15 no.19 pp.5256–5267, 1996

Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002

Gregory J.Brunn[1], Josie Williams[2], Candace Sabers[1], Gregory Wiederrecht[3], John C.Lawrence,Jr[4] and Robert T.Abraham[1,2,5]

Departments of [1]Pharmacology and [2]Immunology, Mayo Clinic, Rochester, MN 55905, [3]Department of Immunology Research, Merck Research Laboratories, Rahway, NJ 07065 and [4]Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St Louis, MO 63110, USA

[5]Corresponding author

The immunosuppressant, rapamycin, inhibits cell growth by interfering with the function of a novel kinase, termed mammalian target of rapamycin (mTOR). The putative catalytic domain of mTOR is similar to those of mammalian and yeast phosphatidylinositol (PI) 3-kinases. This study demonstrates that mTOR is a component of a cytokine-triggered protein kinase cascade leading to the phosphorylation of the eukaryotic initiation factor-4E (eIF-4E) binding protein, PHAS-1, in activated T lymphocytes. This event promotes G_1 phase progression by stimulating eIF-4E-dependent translation initiation. A mutant YAC-1 T lymphoma cell line, which was selected for resistance to the growth-inhibitory action of rapamycin, was correspondingly resistant to the suppressive effect of this drug on PHAS-1 phosphorylation. In contrast, the PI 3-kinase inhibitor, wortmannin, reduced the phosphorylation of PHAS-1 in both rapamycin-sensitive and -resistant T cells. At similar drug concentrations (0.1–1 μM), wortmannin irreversibly inhibited the serine-specific autokinase activity of mTOR. The autokinase activity of mTOR was also sensitive to the structurally distinct PI 3-kinase inhibitor, LY294002, at concentrations (1–30 μM) nearly identical to those required for inhibition of the lipid kinase activity of the mammalian p85–p110 heterodimer. These studies indicate that the signaling functions of mTOR, and potentially those of other high molecular weight PI 3-kinase homologs, are directly affected by cellular treatment with wortmannin or LY294002.

Keywords: cell cycle/phosphatidylinositol-3-kinase/translation/rapamycin/wortmannin

Introduction

The bacterially derived macrolide esters, FK506 and rapamycin, are potent immunosuppressants with established or predicted therapeutic applications in the prevention of organ allograft rejection and in the treatment of autoimmune diseases. In spite of their structural similarity, FK506 and rapamycin suppress immune responses by interfering with distinct steps in the antigen-triggered T-cell activation program (Abraham and Wiederrecht, 1996). Treatment of resting T helper cells with FK506 interrupts an antigen-induced signaling pathway leading to the production of interleukin-2 (IL-2) and other cytokines. In contrast, rapamycin suppresses the proliferative response of activated T lymphoblasts to IL-2 and other growth factors. The growth-inhibitory effects of rapamycin are not limited to T cells, as this drug inhibits the proliferation of many mammalian cell types, as well as that of yeast cells. These observations suggest that rapamycin targets a highly conserved component of the cell cycle regulatory machinery.

The mechanism underlying the inhibitory effect of rapamycin on G_1 to S phase progression has been investigated in both yeast and mammalian model systems. The pharmacologic actions of rapamycin are dependent on interaction of this drug with its major cytosolic receptor, the 12 kDa FK506 binding protein (FKBP12). It is the FKBP12·rapamycin complex, rather than the drug itself, that represents the proximate inhibitor of cell growth. Candidate ligands for this immunophilin·drug complex were first identified through genetic screens in *Saccharomyces cerevisiae* (Heitman *et al.*, 1991; Cafferkey *et al.*, 1993; Kunz *et al.*, 1993; Helliwell *et al.*, 1994). A search for mutations that rendered yeast cells resistant to the antifungal activity of rapamycin yielded two novel and highly related *T*arget *o*f *R*apamycin genes, *TOR1* and *TOR2*. The *TOR1* and *TOR2* open reading frames encode large polypeptides containing 2470 and 2474 amino acids, respectively, with 67% overall sequence identity. Subsequent efforts by several independent groups led to the biochemical purification and molecular cloning of a single FKBP12·rapamycin binding protein from mammalian tissues (Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995). The amino acid sequence of this protein was >40% identical to the yeast proteins, TOR1p and TOR2p, indicating that a mammalian TOR (mTOR) homolog had been identified. The highest degree of sequence identity among all three TOR proteins resides in their carboxy-terminal regions, which bear significant similarity to the phosphotransferase domains of phosphatidylinositol (PI) 3-kinases. The presence of the PI 3-kinase-related catalytic domain fueled speculation that the TOR proteins function as lipid or protein kinases *in vivo*.

The first insights into the functions of the TOR proteins were also provided by genetic studies in yeast, which indicated that TOR1p and TOR2p supply both overlapping and distinct growth-regulatory activities (Kunz *et al.*, 1993; Cafferkey *et al.*, 1994; Helliwell *et al.*, 1994). Disruption of the *TOR1* locus results in slow growth in nutritious medium, whereas a *TOR2* disruption causes cell cycle-independent lethality. A double disruption of *TOR1* and *TOR2* leads to cell death in G_1 phase. These results

suggest that *TOR1* and *TOR2* provide a redundant activity needed for G_1 progression, while *TOR2* supplies an additional, unique activity required for cell viability. Both the cell cycle-regulatory and essential functions of the yeast TOR proteins are dependent on their PI 3-kinase-related catalytic domains (Helliwell *et al.*, 1994). Interestingly, the kinase domains but not the amino-terminal domains of TOR1p and TOR2p are interchangeable, suggesting that the amino-terminal regions mediate the non-overlapping activities of these proteins. Rapamycin treatment arrests yeast cell growth in G_1 phase without lethality, which supports the working model that the immunophilin·drug complex selectively blocks the G_1 progression functions of TOR1p and TOR2p, but leaves the essential activity of TOR2p intact (Zheng *et al.*, 1995; Hall, 1996).

In the presence of rapamycin, IL-2-stimulated T cells accumulate in mid to late G_1 phase (Dumont *et al.*, 1990; Morice *et al.*, 1993; Terada *et al.*, 1993), suggesting that a TOR protein also participates in the regulation of G_1 to S phase progression in lymphoid cells. The G_1 phase growth arrest state induced by rapamycin is characterized by the intracellular accumulation of catalytically inactive G_1 cyclin–cyclin-dependent kinase (cdk) complexes (Morice *et al.*, 1993; Nourse *et al.*, 1994). This phenotype is explained at least in part by the failure of drug-treated T cells to down-regulate the cdk inhibitor protein, $p27^{Kip1}$, in response to IL-2 stimulation (Nourse *et al.*, 1994). Although these results implicate mTOR in the regulation of $p27^{Kip1}$ protein levels during G_1 phase, the intermediate transducer(s) in this pathway remains unknown. Numerous studies have demonstrated that rapamycin blocks the mitogen-induced phosphorylation and catalytic activation of p70 S6 kinase in both lymphoid and non-lymphoid cells (Calvo *et al.*, 1992; Chung *et al.*, 1992; Kuo *et al.*, 1992; Price *et al.*, 1992). The inhibitory effect of rapamycin can be overcome by expression of a mutated version of mTOR that fails to bind to the FKBP12·rapamycin complex, indicating that mTOR is an upstream component of the mitogenic signaling pathway leading to p70 S6 kinase activation (Brown *et al.*, 1995). Interestingly, the activation of p70 S6 kinase by growth factors is also suppressed by wortmannin and LY294002 (Cheatham *et al.*, 1994; Karnitz *et al.*, 1995; Monfar *et al.*, 1995), which are considered to be relatively selective inhibitors of classical PI 3-kinases (Powis *et al.*, 1994; Vlahos *et al.*, 1994; Ui *et al.*, 1995). These pharmacologic observations are consistent with the hypothesis that PI 3-kinase and mTOR lie within linear or parallel pathways leading to p70 S6 kinase activation (Downward, 1994). However, an alternative interpretation is that wortmannin and LY294002 both target the PI 3-kinase-related catalytic domain of mTOR, and that the inhibition of p70 S6 kinase reflects a direct action on mTOR itself.

A second, possibly related, mitogenic response that appears to be governed by mTOR is the stimulation of protein synthesis, specifically the process of translation initiation (Hershey, 1991; Sonenberg, 1993). The rate-limiting step in the translation of certain mRNAs, particularly those with a high degree of secondary structure in their 5′-untranslated regions (5′-UTRs), is the binding of eukaryotic initiation factor-4E (eIF-4E) to the m^7(5′)G-ppp(5′)N (where N is any nucleotide) cap located at the 5′ termini. In quiescent cells, the ability of eIF-4E to interact with eIF-4γ, an essential step in the formation of the active initiation complex, is tonically suppressed by the inhibitor protein, PHAS-1 (Haghighat *et al.*, 1995; Mader *et al.*, 1995). Growth factor stimulation leads to a rapid increase in the phosphorylation of PHAS-1, which decreases its binding affinity for eIF-4E, and thereby relieves the inhibitory constraint on eIF-4E-dependent translation initiation (Lin *et al.*, 1994; Pause *et al.*, 1994). In adipocytes, aortic smooth muscle cells and fibroblasts, the phosphorylation of PHAS-1 and its dissociation from eIF-4E are blocked by rapamycin treatment (Graves *et al.*, 1995; Lin *et al.*, 1995; Beretta *et al.*, 1996). These results imply that mTOR serves as a positive regulator of cap-dependent translation initiation in non-lymphoid cells. Thus, mTOR may participate in a novel regulatory pathway that controls the translation of proteins critical for the progression of mitogen-stimulated cells from G_1 into S phase.

Here, we demonstrate that IL-2 provokes a rapid increase in the phosphorylation of PHAS-1 in activated T lymphocytes. This response is suppressed by pre-treatment of the cells with either rapamycin or wortmannin. Genetic evidence obtained with T lymphoma cell somatic mutants supports the conclusion that mTOR function is required for the phosphorylation of PHAS-1 in T lymphocytes. Furthermore, we show that two commonly used PI 3-kinase inhibitors, wortmannin and LY294002, also inhibit the kinase activity of mTOR. These findings raise a cautionary note concerning the presumed specificity of these drugs as inhibitors of PI 3-kinase-dependent responses in intact cells. On the other hand, these drugs may be useful lead compounds for the development of more selective inhibitors of mTOR and other PI 3-kinase-related kinases in mammalian cells.

Results

Inhibition of PHAS-1 phosphorylation by rapamycin and wortmannin

Recent reports that insulin- and serum-induced PHAS-1 phosphorylation is sensitive to rapamycin (Graves *et al.*, 1995; Lin *et al.*, 1995; Beretta *et al.*, 1996) prompted us to examine the impact of this immunosuppressant on the phosphorylation state of PHAS-1 in a clinically relevant target cell, i.e. the IL-2-stimulated T lymphocyte. To this end, murine IL-2-dependent CTLL-2 cells were deprived of cytokines, and then were restimulated with recombinant IL-2, in the presence or absence of rapamycin. Detergent-free cell extracts were boiled, and soluble proteins were separated by SDS–PAGE and immunoblotted with anti-PHAS-1 antibodies. In CTLL-2 cells, the antibodies detected three bands of immunoreactive PHAS-1, which reflect different phosphorylation states of this protein (Lin *et al.*, 1994) (Figure 1A). Stimulation of the growth factor-starved cells with IL-2 provoked a net loss of the fastest migrating, hypophosphorylated form of PHAS-1, and a concomitant increase in the abundance of more slowly migrating bands corresponding to more highly phosphorylated PHAS-1. Treatment of the cells with 1 nM rapamycin prior to IL-2 stimulation blocked the hyperphosphorylation of PHAS-1, and strongly increased the intensity of the most rapidly migrating band corresponding to hypophosphorylated PHAS-1. The IL-2-induced phosphorylation of

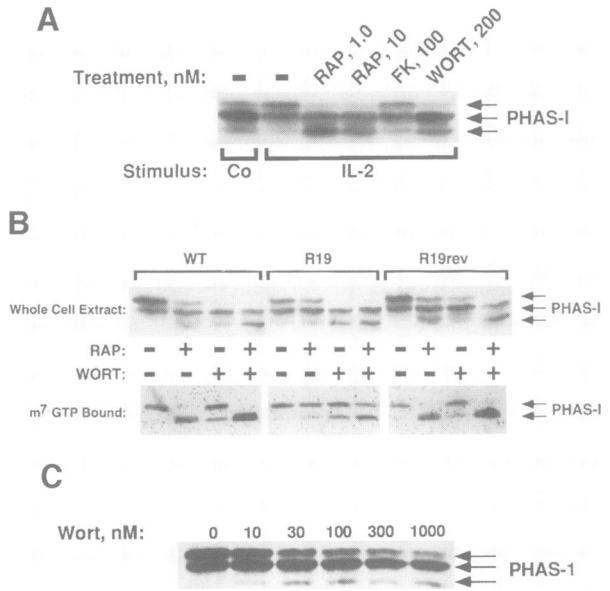

Fig. 1. Inhibition of PHAS-1 phosphorylation in T-cells. (**A**) Factor-deprived CTLL-2 cells were pre-treated for 1 h with no drug (–) or with the indicated concentration of rapamycin (RAP) or wortmannin (WORT). Cells were stimulated for 10 min with medium only (Co) or with 50 U/ml IL-2. The cells were lysed, and heat-stable proteins were resolved by SDS–PAGE and immunoblotted with anti-PHAS-1 antibodies. (**B**) Wild-type YAC-1 cells (WT), rapamycin-resistant R19 cells or rapamycin-sensitive revertant clone R19rev cells were treated for 1 h with 1 nM rapamycin (RAP) or 200 nM wortmannin (WORT). Upper panel: heat-stable proteins were prepared from whole cell extracts as described in (A). Lower panel: detergent-free cell extracts were precipitated with m^7GTP–Sepharose, bound proteins were separated by SDS–PAGE, and PHAS-1 isoforms were detected by immunoblotting. (**C**) Wild-type YAC-1 T cells were pre-treated for 1 h with the indicated concentrations of wortmannin (Wort), and heat-stable cellular proteins were immunoblotted with anti-PHAS-1 antibodies as described above.

PHAS-1 was unaffected by 100 nM FK506, suggesting that the inhibition of PHAS-1 phosphorylation was due specifically to the formation of the FKBP12·rapamycin complex.

Previous studies demonstrated that the mitogen-induced phosphorylation and activation of p70 S6 kinase was sensitive to the PI 3-kinase inhibitor, wortmannin, as well as to rapamycin (Cheatham *et al.*, 1994; Karnitz *et al.*, 1995; Monfar *et al.*, 1995). Moreover, the carboxy-terminal catalytic domain of mTOR bears a higher degree of sequence homology to the catalytic domains of PI 3-kinases than to those of conventional protein kinases. Based on these observations, we postulated that wortmannin might also inhibit irreversibly the catalytic activity of the mTOR. This model predicts that wortmannin, like rapamycin, will block the IL-2-dependent phosphorylation of PHAS-1 in T cells. This was indeed the case, as pre-treatment of CTLL-2 cells with 200 nM wortmannin dramatically reduced the phosphorylation of PHAS-1 induced by subsequent exposure of the cells to IL-2 (Figure 1A).

Several earlier reports characterized a panel of rapamycin-resistant subclones of the YAC-1 T lymphoma cell line (Dumont *et al.*, 1994, 1995; Sabers *et al.*, 1995). Both genetic and biochemical data argue that the resistant clones, including the clone R19 cells used in the present studies, harbor a mutation that results in the expression of a functional mTOR with a lower affinity for the FKBP12·rapamycin complex. To substantiate further the hypothesis that mTOR is an upstream regulator of PHAS-1 phosphorylation, we compared the effects of rapamycin and wortmannin on this response in wild-type YAC-1 cells, clone R19 cells and in a drug-sensitive revertant clone, R19rev, derived from the original R19 population (Sabers *et al.*, 1995). In these studies, exponentially growing YAC-1 clones were treated with the drugs for 1 h prior to cell lysis. Two approaches were used to assess the phosphorylation state of PHAS-1. First, whole cell extracts were immunoblotted directly with anti-PHAS-1 antibodies to examine the total intracellular pool of PHAS-1. Second, parallel aliquots of the same extracts were incubated with m^7GTP–Sepharose to precipitate eIF-4E and associated forms of PHAS-1 (Lin *et al.*, 1994). Both methods of analyzing the phosphorylation state of PHAS-1 yielded qualitatively similar results (Figure 1B). In the absence of drug, all three YAC-1 cell lines contain hyperphosphorylated forms of PHAS-1 corresponding to the upper two bands of immunoreactive protein found in IL-2-stimulated CTLL-2 cells (Figure 1B, upper panel). The uppermost band in this immunoblot represents the most highly phosphorylated form of PHAS-1, which does not bind to eIF-4E, and, therefore, is not found in 7-methyl GTP (m^7GTP)–Sepharose precipitates (Figure 1B, lower panel and data not shown). In drug-sensitive wild-type or clone R19rev cells, rapamycin exposure strongly reduced the amount of hyperphosphorylated PHAS-1 detected in whole cell immunoblots, and concomitantly increased the abundance of the most rapidly migrating, dephosphorylated form of PHAS-1. The drug-induced increase in the level of dephosphorylated PHAS-1 is seen most clearly in immunoblots of the m^7GTP–Sepharose precipitates from these cell extracts. In contrast, rapamycin caused no significant reduction in PHAS-1 phosphorylation in drug-resistant clone R19 cells, which strongly suggests that this response is a consequence of the interaction of the immunophilin·drug complex with mTOR.

As was observed in CTLL-2 cells, wortmannin treatment resulted in a net dephosphorylation of PHAS-1 in wild-type YAC-1 T lymphoma cells (Figure 1B). The mechanism of action of wortmannin is clearly distinct from that of rapamycin, as wortmannin treatment provoked the accumulation of hypophosphorylated PHAS-1 in both rapamycin-sensitive and -resistant YAC-1 clones. Although alternative interpretations are possible (see Discussion), these results are consistent with the notion that wortmannin covalently modifies the mTOR catalytic domain by a mechanism analogous to that described for the p110 subunit of PI 3-kinase (Wymann *et al.*, 1996). Previous results from this laboratory indicate that exposure of intact T cells to 30 nM wortmannin results in maximal inhibition of the lipid kinase activity of the p85–p110 heterodimer (Karnitz *et al.*, 1995). To assess the sensitivity of the PHAS-1 phosphorylation pathway to wortmannin, wild-type YAC-1 cells were treated for 1 h with 0–1000 nM wortmannin, and the level of PHAS-1 phosphorylation was examined by immunoblot analysis. Although this assay is qualitative, the results suggest that maximal inhibition of PHAS-1 phosphorylation in intact T cells occurs at wortmannin concentrations >300 nM (Figure 1C). Thus, the wortmannin-sensitive target in the PHAS-1

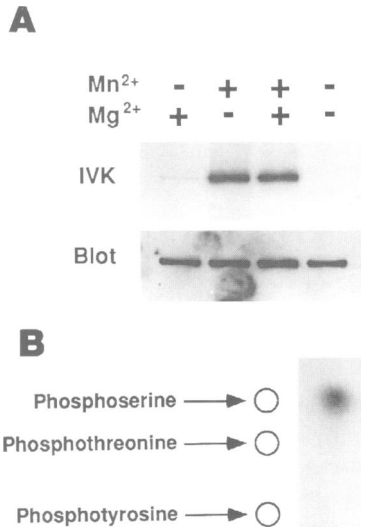

Fig. 2. Autokinase activity of brain-derived mTOR. (**A**) Rat brain extracts (1 mg protein/sample) were incubated with glutathione–agarose-bound GST–FKBP12 in the presence of 10 μM rapamycin. The washed precipitates were suspended in kinase buffer containing [γ-^{32}P]ATP without the indicated divalent cation (–) or with 10 mM Mn^{2+} and/or 10 mM Mg^{2+} (+). Upper panel: proteins from the *in vitro* kinase (IVK) assay were resolved by SDS–PAGE, transferred to Immobilon-P membranes and radiolabeled mTOR was visualized by autoradiography. Lower panel: the same samples were then immunoblotted (Blot) with polyclonal anti-mTOR antibodies. (**B**) Phosphoamino acid analysis. The radiolabeled bands from (A) were excised from the membrane, subjected to acid hydrolysis, and the hydrolysates were separated by cellulose thin-layer chromatography. The migration of unlabeled phosphoamino acid standards is indicated by circles.

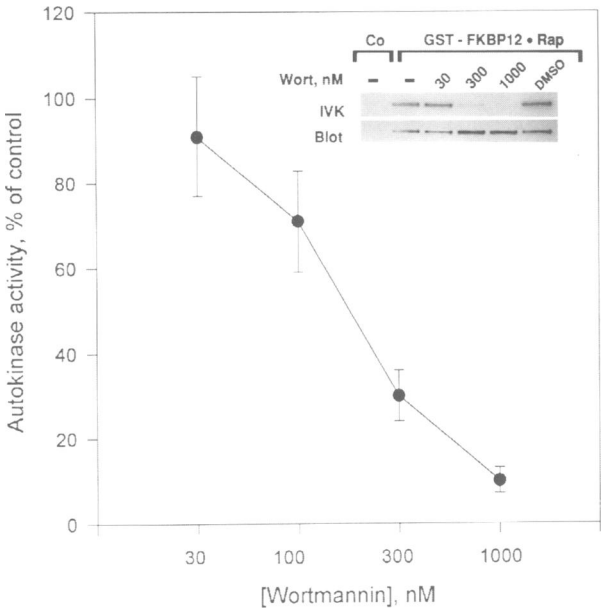

Fig. 3. Inhibition of mTOR autokinase activity by wortmannin. Rat brain-derived mTOR was isolated by precipitation with immobilized GST–FKBP12·rapamycin (Rap). Control (Co) precipitates were prepared in an identical fashion, except that rapamycin was omitted from the precipitation step. The precipitated proteins were washed and treated for 1 h with the indicated concentrations of wortmannin (Wort) or with the drug vehicle DMSO only. Kinase activity was assayed in the presence of Mn^{2+} and [γ-^{32}P]ATP, and $^{32}P_i$ incorporation into mTOR was quantitated with an Ambis image acquisition and analysis system. Cumulative counts were normalized to solvent vehicle (DMSO) controls, and were plotted as mean percentages of control ± standard deviations from three independent trials. The inset shows a representative autoradiograph and immunoblot from a single experiment.

phosphorylation pathway appears significantly less sensitive to wortmannin than is the lipid kinase activity of the p110 subunit of PI 3-kinase.

Inhibition of mTOR autokinase activity by wortmannin

In spite of the sequence homology to PI 3-kinase, we and others (Brown *et al.*, 1995) have failed to show that lipid kinase activity is an intrinsic property of the mTOR catalytic domain. Experiments were therefore performed to determine whether mTOR was capable of functioning as a protein kinase. In the initial studies, we purified mTOR from rat brain extracts using the FKBP12·rapamycin affinity chromatography technique described previously (Sabers *et al.*, 1995). The GST–FKBP12·rapamycin precipitates were washed under stringent conditions, and then assayed for protein kinase activity in the presence of [γ-^{32}P]ATP. In the presence of Mg^{2+} as the only divalent cation, a relatively small amount of radiolabeled phosphate was incorporated into the FKBP12·rapamycin-bound mTOR (Figure 2A). The level of mTOR phosphorylation was greatly enhanced by the addition of Mn^{2+} to the kinase buffer. Phosphoamino acid analysis of the radiolabeled mTOR bands revealed that the phosphate was incorporated exclusively into serine residues (Figure 2B). These results confirm the earlier report that recombinant mTOR exhibits serine-specific autokinase activity *in vitro* (Brown *et al.*, 1995).

Subsequent studies tested the hypothesis that wortmannin inhibits the kinase activity of brain-derived mTOR. The mTOR was again isolated by precipitation with immobilized FKBP12·rapamycin complexes, and the bound protein was treated with various concentrations of wortmannin. Free drug was removed by washing the precipitates, and the samples were incubated in kinase buffer containing Mg^{2+}, Mn^{2+} and [γ-^{32}P]ATP. Pre-treatment of the precipitated mTOR with 30–1000 nM wortmannin resulted in a progressive decrease in the level of mTOR phosphorylation in subsequent kinase assays (Figure 3). The concentration of wortmannin needed to inhibit the phosphorylation of mTOR by 50% (IC_{50}) is ~200 nM. Assuming that the phosphate incorporated into mTOR is due entirely to autophosphorylation, these results suggest that the phosphotransferase activity of mTOR is ~100-fold less sensitive to wortmannin than is the phosphoinositide kinase activity of the mammalian p85–p110 heterodimer.

A recent report has clarified the mechanism of PI 3-kinase inhibition by wortmannin (Wymann *et al.*, 1996). Drug binding to the p110 subunit of PI 3-kinase initially is competitive with respect to ATP, indicating that wortmannin interacts with the ATP binding site of this enzyme. Once positioned in the active site, wortmannin irreversibly modifies the enzyme via electrophilic attack on a lysine residue required for the phosphotransferase reaction. If wortmannin inhibits mTOR autokinase activity by a similar mechanism, then the addition of an excess of a small molecule nucleophile, such as dithiothreitol (DTT), to

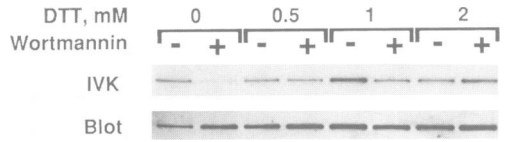

Fig. 4. Dithiothreitol (DTT) antagonizes the inhibitory effect of wortmannin on mTOR autokinase activity. Rat brain-derived mTOR was purified by precipitation with immobilized GST–FKBP12 in the presence of rapamycin. The washed precipitates were treated for 1 h with drug vehicle only (–) or 1 μM wortmannin (+) in the presence of the indicated concentrations of DTT. The *in vitro* kinase (IVK) activity in each sample was determined in the presence of Mn^{2+} and [γ-^{32}P]ATP. Upper panel: after SDS–PAGE, the proteins were transferred to an Immobilon-P membrane, and incorporation of radioactivity into mTOR was detected by autoradiography. Lower panel: the same membrane was immunoblotted with polyclonal anti-mTOR antibodies to determine the total amount of mTOR protein in each lane.

the kinase buffer should interfere with the irreversible modification of mTOR. As shown in Figure 4, DTT, at concentrations as low as 0.5 mM, virtually abrogated the ability of wortmannin to inhibit mTOR autokinase activity. In similar experiments, we found that a low concentration (0.1% w/v) of the non-ionic detergents NP-40 or Triton X-100 also protected mTOR from irreversible modification by wortmannin (data not shown). The protective effects of DTT and non-ionic detergents provide a rational explanation for the failure of earlier investigators to detect a suppressive action of wortmannin on mTOR autokinase activity (Brown *et al.*, 1995).

Radiolabeling of mTOR with [^{3}H]wortmannin

To define further the mechanism of action of wortmannin, we incubated rat brain high-speed supernates with 200 nM 17 β-hydroxy-[^{3}H]wortmannin in the absence of detergent and reducing agents. The labeled extracts were either resolved directly by SDS–PAGE, or were precipitated with glutathione–agarose beads coupled to the GST–FKBP12·rapamycin complex prior to electrophoretic separation of the bound proteins. Surprisingly, [^{3}H]wortmannin labeled relatively few proteins in the crude supernates (Figure 5A). The major doublet located above the 97 kDa marker represents isoforms of the p110 subunit of PI 3-kinase, both of which can be co-precipitated with anti-p85 antibodies (data not shown). Three additional radiolabeled proteins migrated above the 200 kDa marker, and the middle band co-migrated with the radiolabeled mTOR precipitated by the GST–FKBP12·rapamycin affinity matrix. The incorporation of radiolabel into all five major bands was competitively inhibited by the addition of 1 μM cold wortmannin to the labeling reaction. These results demonstrate that mTOR is covalently modified by wortmannin at drug concentrations that inhibit the autokinase activity of this enzyme *in vitro*.

Although the above studies documented that wortmannin binds covalently to mTOR, it remained possible that the drug fortuitously interacted with a nucleophilic residue located outside the ATP binding site. Consequently, we determined whether the covalent modification of mTOR by [^{3}H]wortmannin could be inhibited by an excess of the non-hydrolyzable ATP analog, adenosine 5′-O-(3-thiotriphosphate) (ATPγS). High-speed supernates from rat brain were incubated with [^{3}H]wortmannin in the absence or presence of increasing concentrations of ATPγS.

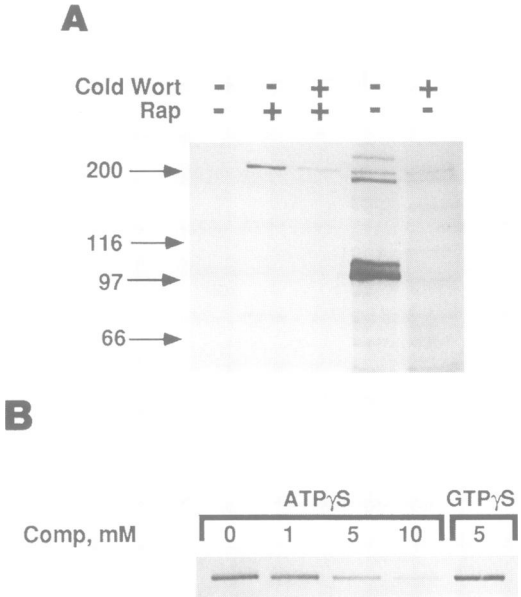

Fig. 5. Radiolabeling of mTOR with [^{3}H]wortmannin. (**A**) Cold competition assay. Rat brain extracts were incubated with 200 nM β-hydroxy-[^{3}H]wortmannin in the absence (–) or presence (+) of 1 μM unlabeled wortmannin (Cold Wort). The labeled extracts were then incubated with 100 μg of GST–FKBP12 coupled to glutathione–agarose in the absence (–) or presence (+) of 10 μM rapamycin (RAP). The precipitated proteins (lanes 1–3) or 100 μg of the crude extract (lanes 4–5) were mixed with sample buffer and separated by SDS–PAGE. After treatment with EN^{3}HANCE, the gel was dried, and radiolabeled proteins were detected by fluorography at –70°C. (**B**) Competitive inhibition of wortmannin binding to mTOR by a non-hydrolyzable ATP analog. Rat brain extracts were incubated with 200 nM [^{3}H]wortmannin in the presence of the indicated concentrations of ATPγS or GTPγS (Comp). The soluble mTOR was isolated by precipitation with glutathione–agarose-bound GST–FKBP12·rapamycin, and [^{3}H]wortmannin-labeled mTOR was analyzed as described in (A).

The radiolabeled mTOR was then isolated from the supernates by binding to GST–FKBP12·rapamycin, and the precipitated proteins were resolved by SDS–PAGE. The radiolabeling of mTOR by [^{3}H]wortmannin was strongly suppressed by the inclusion of ATPγS in the labeling reaction, whereas the non-hydrolyzable GTP analog, guanosine 5′-O-(3-thiotriphosphate) (GTPγS), had no detectable effect on this reaction (Figure 5B). A parallel immunoblot experiment confirmed that the ATPγS pre-treatment had no effect on the recovery of mTOR by the GST–FKBP12·rapamycin affinity matrix (data not shown). These results indicate that wortmannin inhibits mTOR autokinase activity by modifying the ATP binding site of this enzyme. Moreover, the inability of GTPγS to block the covalent interaction between wortmannin and mTOR suggests that ATP, but not GTP, serves as a phosphate donor for the substrate phosphorylation reaction(s) catalyzed by mTOR *in vivo*.

Effect of wortmannin on the autokinase activity of recombinant mTOR

To verify that the inhibitory effect of wortmannin on mTOR phosphorylation was due to the loss of mTOR kinase activity, we expressed the full-length mTOR cDNA in Sf9 cells using the baculovirus expression system. The recombinant mTOR was tagged at the carboxy-terminus with amino acids comprising the epitope recognized by

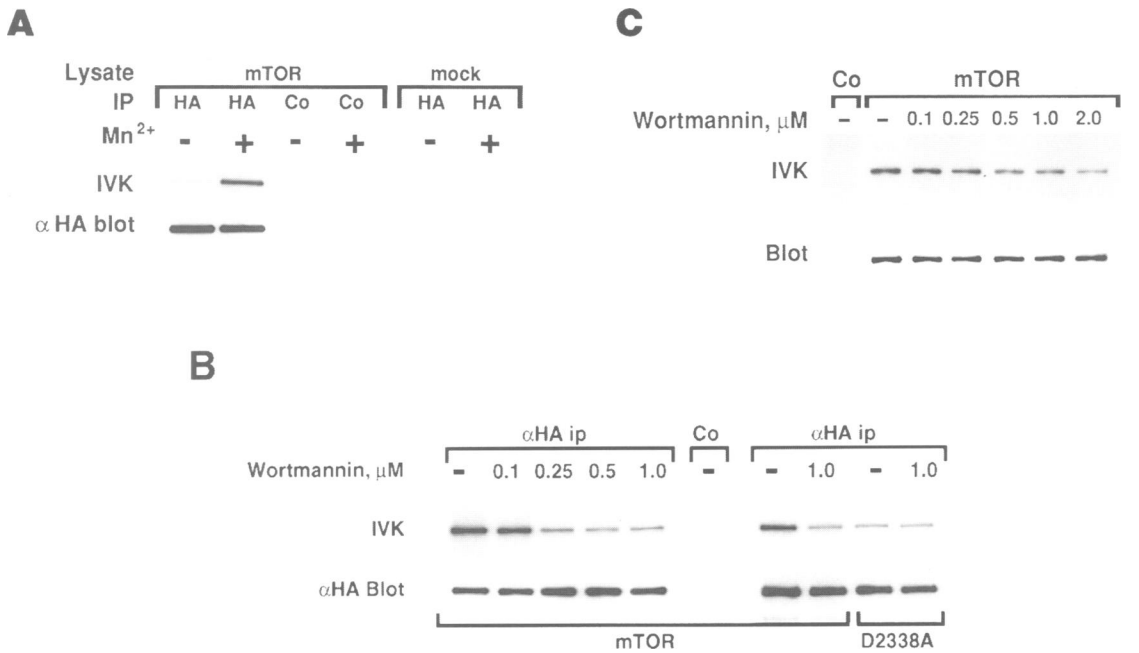

Fig. 6. Effect of wortmannin on the autokinase activity of recombinant mTOR. (**A**) Effect of divalent cations on mTOR kinase activity. Extracts from Sf9 cells infected with non-recombinant (mock) or HA-tagged mTOR baculovirus constructs were immunoprecipitated (IP) with either anti-HA or isotype-matched control (Co) monoclonal antibodies. The washed immunoprecipitates were assayed for kinase activity in the presence of [γ-^{32}P]ATP and Mg^{2+}, plus or minus Mn^{2+}. Radiolabeled, recombinant mTOR was visualized by autoradiography (IVK, upper panel) and by immunoblot analysis using anti-HA serum (αHA blot, lower panel) as in Figure 2. (**B**) Inhibition of mTOR kinase activity by wortmannin. Anti-HA (αHA ip) or isotype-matched control monoclonal antibody (Co) immunoprecipitates from Sf9 cell lysates containing HA-tagged, wild-type mTOR or mutant mTOR containing the D2338→A2338 substitution (D2338A) were treated with the indicated concentrations of wortmannin for 1 h. The washed immunoprecipitates were incubated with [γ-^{32}P]ATP in the presence of Mn^{2+} and analyzed as in (A). (**C**) Inhibition of mTOR kinase activity by treatment of intact cells with wortmannin. Sf9 cells were infected with wild-type baculovirus (Co) or recombinant baculovirus encoding HA-tagged mTOR (mTOR). The infected cells were treated for 1 h with the indicated concentrations of wortmannin, the cells were lysed and kinase assays were performed in the presence of [γ-^{32}P]ATP and Mn^{2+}.

anti-hemagglutinin (HA) antibodies. As shown in Figure 6A, anti-HA antibody immunoprecipitates from insect cells infected with the recombinant baculovirus, but not control baculovirus, contained a single immunoreactive protein that migrated at the predicted molecular mass of full-length mTOR. Like rat brain-derived mTOR, the recombinant mTOR phosphorylated itself on a serine residue(s) in the presence of Mg^{2+}-ATP, and this kinase activity was markedly stimulated by the addition of Mn^{2+} to the kinase buffer. In preliminary studies, we noted that the anti-HA immunoprecipitates from mTOR-containing insect cell lysates were contaminated with a co-precipitating protein kinase(s) that transphosphorylated the mTOR during the *in vitro* kinase reaction. This problem was only partially overcome by preparing and washing the immunoprecipitates under relatively high stringency conditions (see Materials and methods). Hence, the possible contribution of this activity to the phosphorylation of recombinant mTOR should be considered when interpreting the results of the immune complex kinase assays described below.

To determine whether the autokinase activity of mTOR was inhibitable by wortmannin, extracts were prepared from Sf9 cells expressing wild-type mTOR or mutant mTOR bearing an aspartic acid to alanine substitution (D2338→A2338) in the catalytic domain. Mutation of the homologous residue in the yeast PI 3-kinase encoded by *VPS34* has been shown previously to abrogate catalytic activity (Stack and Emr, 1994). The wild-type and the D2338→A2338 mutant mTOR were immunoprecipitated from Sf9 cell extracts with anti-HA antibody, and the immunoprecipitates were treated with varying concentrations of wortmannin. The immunoprecipitates were then washed, and autokinase activity was determined in the presence of Mn^{2+} (Figure 6B). Wortmannin pre-treatment inhibited the phosphorylation of recombinant mTOR at concentrations similar to those required for inhibition of the FKBP12·rapamycin-bound mTOR isolated from rat brain (Figure 6B). The failure of wortmannin to completely block the phosphorylation of mTOR in these assays probably reflects the background level of phosphorylation contributed by the contaminating protein kinase activity mentioned above. This background activity is insensitive to wortmannin, as the basal level of phosphate incorporated into the kinase-inactive, D2338→A2338 mTOR mutant was unaffected by pre-treatment of the immunoprecipitate with 1 μM wortmannin (Figure 6B). Thus, the autokinase activities displayed by both rat brain-derived and recombinant mTOR were inhibited with similar potencies by wortmannin.

Inhibition of mTOR autokinase activity in intact cells

A number of published studies have used wortmannin at concentrations in excess of 100 nM in order to investigate the role of PI 3-kinase in a broad range of cellular processes (see, for example, Ferby *et al.*, 1994; Ding *et al.*, 1995; Skorski *et al.*, 1995). The present findings raise a cautionary note that mTOR function may also be inhibited when cells are exposed to sub- or low-micromolar

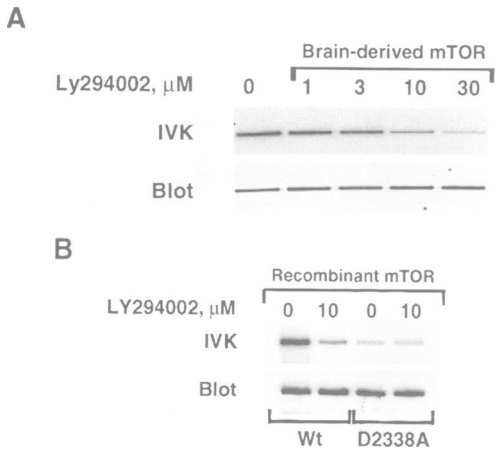

Fig. 7. Inhibition of mTOR autokinase activity by LY294002. (**A**) Autokinase activity of rat brain-derived mTOR. Rat brain extracts were incubated with glutathione–agarose-bound GST–FKBP12 in the presence of rapamycin. The precipitates were washed and suspended in Mn^{2+}-containing kinase buffer supplemented with the indicated concentrations of LY294002. After 10 min at 30°C, [γ-^{32}P]ATP was added, and the samples were incubated for an additional 10 min. The reactions were terminated with sample buffer, and bound proteins were separated by SDS–PAGE. The proteins were transferred onto an Immobilon-P membrane, and radiolabeled mTOR was detected by autoradiography. Incorporation of radioactivity into mTOR was also quantitated with an Ambis imaging system (see text for results). (**B**) Autokinase activity of recombinant mTOR. Extracts from Sf9 insect cells expressing either wild-type (wt) mTOR or a kinase-dead (D2338A) mTOR mutant were immunoprecipitated with anti-HA monoclonal antibody. The washed immunoprecipitates were pre-treated with solvent vehicle only or with 10 μM LY294002, and mTOR autokinase activity was assayed as described in (A).

concentrations of wortmannin. As an initial approach to test this hypothesis, mTOR-expressing insect cells were treated with various concentrations of wortmannin prior to cell lysis. The epitope-tagged mTOR was then immunoprecipitated from the cell extracts, and autokinase activity was assayed as described above. The catalytic activity of mTOR, as measured in immune complex kinase assays, was progressively inhibited by cellular pre-treatment with wortmannin (Figure 6C). The approximate IC_{50} for wortmannin in this assay was 300 nM, which is reasonably similar to the IC_{50} value obtained after direct treatment of FKBP12·rapamycin-bound mTOR with this drug (refer to Figure 3). Thus, these results strongly suggest that treatment of intact cells with submicromolar concentrations of wortmannin leads to the inhibition of mTOR kinase activity *in vivo*.

Inhibition of mTOR autokinase activity by LY294002

Due in part to the chemical instability of wortmannin in biological systems, a structurally unrelated PI 3-kinase inhibitor, LY294002, is being used increasingly as a pharmacologic probe in mammalian cells. Unlike wortmannin, LY294002 reversibly inhibits the lipid kinase activity of the p85–p110 heterodimer, with an IC_{50} of ~3 μM. To determine whether the catalytic domain of mTOR was also targeted by LY294002, FKBP12·rapamycin-bound mTOR was assayed for autokinase activity in the presence of 0–30 μM LY294002. As shown in Figure 7A, LY294002 inhibited the autokinase activity of rat brain-derived mTOR, with an approximate IC_{50} value of 5 μM. The autokinase activity of immunopurified, recombinant mTOR was similarly sensitive to LY294002. Thus, LY294002 effectively targets the catalytic domain of mTOR at concentrations similar to those needed for inhibition of mammalian PI 3-kinases.

Discussion

Studies using rapamycin as a pharmacologic probe have implicated a signaling pathway(s) governed by mTOR in the activation of p70 S6 kinase and the enhancement of eIF-4E-dependent protein synthesis in mitogen-stimulated cells (Graves *et al.*, 1995; Lin *et al.*, 1995; Abraham and Wiederrecht, 1996; Barbet *et al.*, 1996; Beretta *et al.*, 1996). The inhibitory effect of rapamycin on eIF-4E function appears to be mediated through the disruption of a kinase pathway leading to the phosphorylation of the eIF-4E repressor protein, PHAS-1. The present findings further substantiate the hypothesis that mTOR is an upstream component of this mitogen-stimulated pathway in IL-2-responsive and leukemic T lymphocytes. Previous reports indicated that wortmannin, like rapamycin, inhibited the phosphorylation and activation of p70 S6 kinase in growth factor-stimulated cells (Cheatham *et al.*, 1994; Karnitz *et al.*, 1995; Monfar *et al.*, 1995). We now show that rapamycin and wortmannin exert concordant inhibitory effects on the phosphorylation of PHAS-1 in proliferating T lymphocytes. Furthermore, our results indicate that wortmannin, as well as the structurally unrelated PI 3-kinase inhibitor, LY294002, suppress the *in vitro* kinase activity of mTOR at drug concentrations similar to those required for the inhibition of PHAS-1 phosphorylation in intact T cells. Hence, the potential interactions of wortmannnin and LY294002 with mTOR and other PI 3-kinase-related kinases should be considered when these drugs are employed as research tools for the dissection of intracellular signaling pathways.

The presence of the PI 3-kinase homology domain led to early suggestions that mTOR transmitted mitogenic signals through the phosphorylation of phosphoinositides or other lipid substrates. In fact, two reports indicated that PI 4-kinase activity is present in mTOR and yeast TOR2p immunoprecipitates (Cardenas and Heitman, 1995; Sabatini *et al.*, 1995); however, neither study ruled out the possibility that the observed lipid kinase activity was due to a co-precipitating phosphoinositide kinase. Our results concur with those of Brown *et al.* (1995), who demonstrated that mTOR phosphorylates itself on serine residues in immune complex kinase assays. However, it should be noted that autokinase activity does not prove that mTOR functions as a *bona fide* protein kinase *in vivo*. The classification of mTOR as a protein kinase should, therefore, remain provisional until an exogenous polypeptide substrate is identified.

The autokinase activity displayed by mTOR shares at least two characteristics with those attributed previously to mammalian and yeast PI 3-kinases. First, the autophosphorylation of both rat brain-derived and recombinant mTOR is strongly enhanced when Mn^{2+}-[γ-^{32}P]ATP, rather than Mg^{2+}-[γ-^{32}P]ATP, serves as the phosphate donor. The protein kinase activities of yeast VPS34p and the mammalian p110 subunit of PI 3-kinase were similarly stimulated by the addition of Mn^{2+} to the kinase reactions

(Dhand *et al.*, 1994; Stack and Emr, 1994). Second, the protein kinase activities of mTOR, p110 and VPS34p are irreversibly inhibited by nanomolar concentrations of wortmannin. While this manuscript was in preparation, Wymann *et al.* (1996) demonstrated that wortmannin covalently modifies a specific lysine residue (K802) located in the catalytic domain of p110. Mutational analysis indicated that this residue is critical for phosphotransferase activity. The authors also noted that a homologous lysine residue (K2187) resides in the catalytic domain of mTOR, and speculated that mTOR kinase activity might be sensitive to wortmannin. The present results confirm that wortmannin covalently modifies both brain-derived and recombinant mTOR. Furthermore, the fact that ATPγS, but not GTPγS, competitively inhibits the binding of wortmannin to mTOR strongly suggests that an ATP binding site residue, most likely K2187, is targeted by wortmannin.

An unexpected observation is that mTOR retains a significant level of serine-specific autokinase activity when bound to the FKBP12·rapamycin complex. This finding apparently conflicts with an earlier report that exogenously added FKBP12·rapamycin blocks the autokinase activity of recombinant mTOR in immune complex kinase assays (Brown *et al.*, 1995). A possibility that cannot be completely excluded is that FKBP12·rapamycin precipitates from rat brain extracts contain a contaminating protein kinase that transphosphorylates mTOR. However, our pharmacologic data argue strongly that the observed kinase activity is intrinsic to the rat brain-derived mTOR itself. The kinase activity present in the FKBP12·rapamycin precipitates was irreversibly inhibited by wortmannin at drug concentrations identical to those required for covalent modification of the active site of mTOR. The submicromolar concentrations of wortmannin used in these assays do not inhibit the catalytic activities of many conventional protein kinases, including Src, Raf-1, MEK, MAP kinase, $p34^{cdc2}$, $p33^{cdk2}$, p70 S6 kinase or protein kinase C isoforms (Karnitz *et al.*, 1995; Ui *et al.*, 1995; G.Brunn and R.Abraham, unpublished data). Moreover, the kinase activity present in FKBP12·rapamycin–mTOR precipitates is completely insensitive to 0.5 μM staurosporine (G.Brunn and R.Abraham, unpublished data), which inhibits a broad range of conventional protein kinases (Ruegg and Burgess, 1989; Herbert *et al.*, 1990). Although admittedly indirect, the available pharmacologic data support the conclusion that mTOR itself is responsible for the mTOR-phosphorylating activity found in the FKBP12·rapamycin precipitates.

The discrepancy between the previous findings (Brown *et al.*, 1995) and the present results may be explained by differences in the preparations of mTOR examined in these two studies. In the earlier report, recombinant mTOR was immunopurified from insect cell lysates, and the kinase activity of the antibody-bound enzyme was determined in the absence or presence of exogenously added FKBP12·rapamycin. In contrast, we performed our kinase assays with rat brain-derived mTOR purified directly with immobilized FKBP12·rapamycin complexes. In our hands, the brain-derived mTOR phosphorylates itself to a far higher stoichiometry than does the baculovirus-expressed, recombinant kinase. The more robust autokinase activity of the rat brain-derived mTOR may reflect the impact of post-translational modifications (e.g. phosphorylation, homodimerization or subunit association) that do not occur in the baculovirus expression system. It is possible that the higher level of catalytic activity expressed by brain-derived mTOR permits significant autophosphorylation to occur in the presence of the putative inhibitor, FKBP12·rapamycin.

The presence of autokinase activity in FKBP12·rapamycin-bound mTOR, together with the finding that the target protein can be labeled with [^{3}H]wortmannin under these conditions, indicates that the active site of mTOR is not completely occluded by interaction with the immunophilin·drug complex. This scenario is reminiscent of the interaction of FKBP12·FK506 with the calcineurin A subunit, in that the immunophilin·drug complex interferes with the entry of bulky polypeptide substrates into a channel leading to the active site, but does not preclude the entry of the small molecule substrate, *p*-nitrophenylphosphate, into this site (Griffith *et al.*, 1995). A detailed view of the impact of FKBP12·rapamycin on the kinase domain of mTOR awaits the determination of the X-ray structure of the ternary complex of immunophilin, drug and target protein. However, the idea that the kinase domain of mTOR retains at least partial function when bound to FKBP12·rapamycin is substantiated by genetic studies in yeast. In *TOR2*-disrupted yeast strains, a kinase-dead TOR2p mutant does not rescue cell viability, indicating that kinase activity is essential for the viability-promoting function of TOR2p (Zheng *et al.*, 1995). In contrast, treatment of wild-type yeast cells with rapamycin provokes a G_1 phase growth arrest without loss of viability (Barbet *et al.*, 1996; Hall, 1996). These genetic data argue that the FKBP12·rapamycin complex does not induce a functional knockout of the kinase domain of yeast TOR2p *in vivo*.

The observation that both wortmannin and LY294002 target the kinase domain of mTOR provides a plausible explanation for the parallel effects of rapamycin, wortmannin and LY294002 on certain cellular processes. Both drugs inhibit the cytokine-dependent growth of T lymphocytes and myeloid progenitor cell lines by blocking G_1 to S phase progression (Karnitz *et al.*, 1995; L.Karnitz and R.Abraham, unpublished data). As mentioned above, we now know that rapamycin and wortmannin interfere with the signaling pathway(s) responsible for mitogen-induced p70 S6 kinase activation and PHAS-1 phosphorylation. The concordance in terms of mechanism of action is indicated further by the recent observation that treatment of rodent fibroblasts with rapamycin or wortmannin provokes the dephosphorylation of identical serine and threonine residues on p70 S6 kinase (Han *et al.*, 1995). This finding led the authors to conclude that the wortmannin-sensitive and rapamycin-sensitive target proteins lie in the same signaling pathway as p70 S6 kinase. Our results support this proposal, and further suggest that rapamycin and wortmannin converge on the same component of the p70 S6 kinase activation pathway, i.e. mTOR. Although an upstream regulatory role for PI 3-kinase remains a distinct possibility, the present findings do inject a cautionary note concerning the interpretation of earlier data obtained with the PI 3-kinase inhibitors wortmannin and LY294002.

The results of this study strengthen the notion that mTOR functions in the mitogen-induced signaling pathway

leading to the stimulation of eIF-4E-dependent protein synthesis during G_1 phase. An important endpoint of this pathway is the hyperphosphorylation of the eIF-4E binding protein, PHAS-1, which triggers the dissociation of this protein from eIF-4E, and permits assembly of the eIF-4F complex at the mRNA cap site (Lin *et al.*, 1994; Pause *et al.*, 1994). Initial results implicated MAP kinase as the proximate effector of PHAS-1 phosphorylation (Lin *et al.*, 1994). However, subsequent studies demonstrated that PHAS-1 hyperphosphorylation proceeded normally in insulin-stimulated cells treated with a pharmacological inhibitor that completely blocked the activation of MAP kinase (Lin *et al.*, 1995). Using both IL-2-dependent and transformed T cell lines, we have confirmed recent reports (Graves *et al.*, 1995; Lin *et al.*, 1995; Beretta *et al.*, 1996) that rapamycin inhibits the phosphorylation of the eIF-4E binding protein, PHAS-1. In addition, we have shown that YAC-1 T lymphoma cells selected for resistance to the anti-proliferative effect of rapamycin were correspondingly resistant to the inhibitory effect of this drug on PHAS-1 phosphorylation. Earlier results demonstrated that the rapamycin-resistant phenotype of this YAC-1 somatic mutant is due to an alteration resulting in a decrease in the binding affinity of mTOR for the FKBP12·rapamycin complex (Sabers *et al.*, 1995). Thus, the present findings supply genetic evidence to support the hypothesis that mTOR participates in the signaling pathway leading to PHAS-1 phosphorylation in mammalian cells.

Previous reports indicating that wortmannin treatment prevents the activation of p70 S6 kinase (Cheatham *et al.*, 1994; Karnitz *et al.*, 1995; Monfar *et al.*, 1995) prompted us to investigate the impact of wortmannin on growth factor-stimulated PHAS-1 phosphorylation in T cells. We now report that submicromolar concentrations of wortmannin inhibit both the IL-2-stimulated and constitutive phosphorylation of PHAS-1 observed in factor-dependent and transformed T cell lines, respectively. While this manuscript was under review, von Manteuffel *et al.* (1996) reported that insulin-induced phosphorylation of PHAS-1 was suppressed by wortmannin in human embryonic kidney cells. The ability of this drug to inhibit PHAS-1 phosphorylation is consistent with the subsequent finding that wortmannin covalently modifies the PI 3-kinase-related catalytic domain of mTOR. Again, our results do not preclude an upstream role for PI 3-kinase in the PHAS-1 phosphorylation pathway; however, we can conclude that the inhibitory effect of wortmannin on this response is explained, at least in part, by the irreversible inhibition of mTOR kinase activity.

The observation that mTOR supplies a positive signal for eIF-4E function suggests a testable model to explain the mechanism whereby rapamycin interferes with G_1 to S phase progression in mammalian cells. By promoting the dissociation of PHAS-1, the mTOR-dependent signaling pathway stimulates the interaction of eIF-4E with the remaining components of the eIF-4F complex at the mRNA cap site (Sonenberg, 1993). This event promotes the unwinding of secondary structure in the 5′-UTR, and facilitates positioning of the ribosome at the AUG initiation codon. One prediction of this model is that translation initiation on mRNAs bearing extensive secondary structure in their 5′-UTRs of individual mRNAs will be highly dependent on the binding of eIF-4F and, consequently, will be most severely impaired by inhibitors of mTOR function, such as rapamycin and wortmannin. A recent study confirmed that rapamycin interferes selectively with cap-dependent, but not cap-independent, translation in mammalian cells (Beretta *et al.*, 1996). The model that emerges from these findings is that the synthesis of a protein(s) needed for G_1 phase progression is highly cap dependent and, therefore, sensitive to alterations in eIF-4E function induced by growth factors and growth inhibitors, including rapamycin and wortmannin.

The above model is corroborated by recent studies in yeast rendered deficient in TOR protein function by treatment with rapamycin or disruption of the *TOR1* and *TOR2* genes (Barbet *et al.*, 1996). In both cases, haploid yeast cells arrest in early G_1 phase, prior to START, and assume a phenotype characteristic of that found in nutrient-starved cells entering stationary (G_0) phase. The cell cycle arrest is preceded by a profound reduction in protein synthesis, caused largely if not entirely by a defect in translation initiation, which is also controlled by a eIF-4E homolog (the *CDC33* gene product) in yeast. Moreover, introduction of a G_1 cyclin gene (*CLN3*) construct, whose 5′-UTR was modified to bypass the normal dependence on eIF-4E for translation initiation, suppressed rapamycin-induced G_1 growth arrest. These data suggest that the TOR signaling pathway coordinates nutrient status with cell cycle progression in yeast by modulating eIF-4E function and, in turn, the translation of labile regulatory proteins, such as G_1 cyclins. Although this model is certainly provocative, the extent to which the yeast data explain the anti-proliferative effects of rapamycin on hematopoietic cells remains to be determined.

The yeast and mammalian TOR proteins are now recognized as members of a growing family of high molecular weight kinases whose catalytic domains resemble those of PI 3-kinases (for reviews, see Hunter, 1995; Keith and Schreiber, 1995; Zakian, 1995; Abraham, 1996). Other members of this family include the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}), and the *ATM* gene product, which is mutated in the human disorder, ataxia telangiectasia. In contrast to the TOR proteins, which appear to be involved in translational control, the functions of the remaining PI 3-kinase homologs seem to be focused on the maintenance of genomic stability. Given the sequence homology to the PI 3-kinase catalytic domain, it would not be surprising if additional members of this family were covalently modified by wortmannin. Indeed, while the present studies were in progress, Hartley *et al.* (1995) reported that wortmannin inhibited the protein kinase activity of DNA-PK_{cs} with a potency (IC_{50}, 200 nM) identical to that observed with mTOR as the target enzyme. An amino acid sequence alignment with the catalytic domain of mammalian p110 predicts that DNA-PK_{cs} will be modified at K3752 by wortmannin (Wymann *et al.*, 1996). Because the catalytic domains of all PI 3-kinase family members contain a homologous lysine residue, it is likely that non-covalent interactions within the ATP binding site will determine the efficiency with which the reactive carbon (C-20) of wortmannin attacks the nucleophilic ε-amino group of this lysine. Structural modifications of wortmannin might therefore yield drugs with selectivity for individual PI 3-kinase homologs, including mTOR. Such drugs would

greatly facilitate the functional characterization of this novel family of signaling proteins, and might have therapeutic applications as immunosuppressive and anti-cancer agents.

Materials and methods

Cell lines

The murine cytotoxic T cell line, CTLL-2, and the *Spodoptera frugiperda* insect cell line, Sf9, were obtained from American Type Culture Collection (Rockville, MD). CTLL-2 cells were maintained in IL-2-containing growth medium (Abraham *et al.*, 1987). Sf9 cells were cultured in serum-free SF900 II medium (Gibco BRL) at 27°C as previously described (King and Possee, 1992). The YAC-1 T lymphoma cell lines were kindly provided by Dr Francis Dumont (Merck Research Laboratories, Rahway, NJ), and were maintained in RPMI 1640 medium buffered to pH 7.2 with 10 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), and supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 50 μM 2-mercaptoethanol. The derivation of the rapamycin-resistant and -sensitive YAC-1 subclones has been described previously (Dumont *et al.*, 1994).

Reagents, fusion proteins and antibodies

Recombinant human IL-2 was generously provided by Hoffman-LaRoche Inc. (Nutley, NJ). Wortmannin was obtained from the Sigma Chemical Company (St Louis, MO), and was dissolved in dimethylsulfoxide (DMSO) to a final drug concentration of 1.2 mM. LY294002 was kindly provided by Dr Chris Vlahos (Lilly Research Laboratories, Indianapolis, IN), and was diluted into aqueous medium from a 20 mM stock solution in DMSO. Rapamycin stock solutions (10 mM) were prepared in ethanol. All drug stock solutions were aliquoted and stored in the dark at −70°C. ATPγS and GTPγS were purchased from Sigma and were dissolved in water immediately prior to use. Bovine serum albumin (BSA, fraction V) was also purchased from Sigma. Unless otherwise indicated, all remaining reagents were from standard commercial sources.

GST–FKBP12 fusion protein was expressed in *Escherichia coli* and purified as described (Sabers *et al.*, 1995). Antiserum directed against the amino acid sequence LELAVPG (residues 2136–2142) found in rodent (and human) mTOR (Brown *et al.*, 1994; Sabers *et al.*, 1995) was generated by immunizing rabbits with a keyhole limpet hemocyanin-coupled peptide. In preliminary immunoblot analyses, this antiserum recognized a protein band that displayed the electrophoretic mobility predicted for full-length mTOR. The immunoreactivity was inhibited specifically by the inclusion of 5 μM immunizing peptide in the immunoblotting solution. Monoclonal antibody 12CA5, which recognizes the HA epitope of influenza virus, and rabbit polyclonal anti-HA antibodies were purchased from BAbCo, Inc. (Richmond CA). The monoclonal anti-HA antibody was used to immunoprecipitate epitope-tagged versions of mTOR, whereas polyclonal anti-HA antibodies were used to immunoblot the tagged proteins.

Immunoblot analysis

After preparation of samples as indicated below, the solubilized proteins were separated by SDS–PAGE through 12.5% gels for PHAS-1 immunoblots or through 7.5% gels for mTOR immunoblots. The proteins were transferred electrophoretically (150 V, 1 h) to Immobilon-P membranes (Millipore), which were then blocked overnight in 25 mM Tris–HCl, pH 7.2, 150 mM NaCl, 0.2% w/v Tween 20 (TBST) supplemented with 5% non-fat dry milk (for anti-PHAS-1 immunoblots) or 2% BSA (for anti-mTOR and anti-HA immunoblots). The membranes were probed with the following antisera, diluted in TBST containing 0.1% BSA, as indicated in parentheses: anti-PHAS-1 (1:500), anti-mTOR (1:1000) or HA.11 (1:3000). Immunoreactive proteins were detected with protein A coupled to horseradish peroxidase and the enhanced chemiluminescence detection reagent (Amersham)

PHAS-1 phosphorylation

CTLL-2 cells were deprived of cytokines by culturing 1×10^6 cells/ml for 5 h in serum-free arrest medium (RPMI 1640 medium containing 100 μg/ml BSA, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 10 mM HEPES, pH 7.2). The growth factor-starved cells were pre-treated with the indicated concentrations of rapamycin or wortmannin during the final hour in culture, and then stimulated for 10 min with 50 U/ml rIL-2. Wild-type or mutant YAC-1 clones were resuspended in arrest medium at 2×10^6 cells/ml, and were treated for 1 h with rapamycin or wortmannin at 37°C. The CTLL-2 or YAC-1 cells (2×10^7 cells per sample) were harvested by centrifugation, washed in phosphate-buffered saline (PBS) and resuspended in 300 μl of lysis buffer (50 mM β-glycerophosphate, 50 mM sodium fluoride and 1.5 mM EGTA, pH 7.4) supplemented with 1 mM DTT, 120 nM okadaic acid and 10 μg/ml each of leupeptin, pepstatin and aprotinin. Cells were lysed with four 5 s pulses from a Braun-Sonic microtip sonicator set at 50% full power. Cell lysates were clarified by centrifugation for 10 min at 10 000 *g*. For analysis of the total pool of intracellular PHAS-1, the extracts (200 μg of total protein) were heated for 5 min at 100°C. The boiled extracts were centrifuged to remove insoluble material, and the supernatants were mixed with Laemmli sample buffer for SDS–PAGE and anti-PHAS-1 antibody immunoblotting as described above. To analyze specifically the pool of PHAS-1 bound to eIF-4E, cellular extracts (200 μg total protein) were precipitated with 30 μl of m^7GTP coupled to Sepharose 4B (Pharmacia) (Lin *et al.*, 1994). The precipitated proteins were eluted from the matrix with SDS–PAGE sample buffer and were prepared for immunoblot analysis with anti-PHAS-1 antibodies as described above.

Characterization of rat brain-derived mTOR

Rat brain tissue extracts were prepared as previously described (Sabers *et al.*, 1995). Extracts (1 mg protein/ml) were incubated with 50 μl of glutathione–agarose (Sigma) bound to GST–FKBP12 fusion protein in the presence of 10 μM rapamycin or drug vehicle only. After mixing for 1 h at 4°C, the GST–FKBP12-coupled beads were washed twice with homogenization buffer (50 mM Tris–HCl, 100 mM NaCl, 10% glycerol, pH 7.4) and twice with kinase buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$). Where indicated, the washed precipitates were resuspended in 1 ml of kinase buffer, and were incubated with various concentrations of wortmannin for 1 h at 24°C. To remove unreacted wortmannin, the precipitates were centrifuged and washed once in kinase buffer supplemented with 1 mM DTT. The autokinase activity of the GST–FKBP12-rapamycin-bound mTOR was assayed by suspending the beads in 40 μl of reaction buffer (kinase buffer supplemented with 1 mM DTT, 10 μg/ml each of leupeptin, pepstatin and aprotinin, 20 μCi [γ-^{32}P]ATP and, where indicated, 10 mM $MnCl_2$). The samples were incubated for 10 min at 30°C, and the kinase reactions were terminated by washing the precipitates with 1 ml of ice-cold PBS. Bound proteins were eluted from the glutathione–agarose beads with sample buffer, and were resolved by SDS–PAGE through a 7.5% gel. The separated proteins were transferred to an Immobilon-P membrane for autoradiographic and immunoblot analyses. Incorporation of radioactivity was quantitated with an Ambis detection and analysis system. The membrane-bound, radiolabeled mTOR was subjected to acid hydrolysis and phosphoamino acid analysis as previously described (Kamps and Sefton, 1989).

Expression and characterization of recombinant mTOR

The full-length mTOR cDNA (Sabers *et al.*, 1995) was modified at the 3′ terminus using the polymerase chain reaction (PCR) with oligonucleotide primers designed to eliminate the stop codon and to introduce a *Not*I restriction site in its place. The *Not*I restriction site was used to append a nucleotide sequence encoding the influenza virus HA epitope to the 3′ end of the open reading frame of mTOR. The HA epitope is recognized by monoclonal antibody 12CA5 (BAbCo, Richmond, CA). The HA-tagged mTOR cDNA was cloned into the BaculoGold pAcSG2 vector (Pharmingen, San Diego, CA). The Transformer Mutagenesis Kit (Clontech, Palo Alto, CA) was used to introduce a missense mutation in the mTOR coding sequence, resulting in the substitution of alanine for aspartic acid at residue 2338 in the mTOR catalytic domain. Products derived from the PCR and mutagenesis were sequenced to insure that the surrounding nucleotide sequences had been replicated faithfully in the wild-type and 'kinase-dead' (D2338→A2338) cDNAs.

Recombinant baculoviruses and high-titer viral stocks for infection of Sf9 insect cells were generated according to the BaculoGold protocol (Pharmingen). To determine the effect of wortmannin on the autokinase activity of recombinant mTOR, Sf9 cells (4×10^6 cells per 60 mm dish) were infected with the appropriate virus, and then were incubated for 36 h at 27°C, and the adherent cells were scraped from the dishes into homogenization buffer supplemented with 15 mM β-glycerophosphate, 250 mM NaCl, 2 mM EDTA, 10 μg/ml each of leupeptin, pepstatin and aprotinin, 1 mM phenylmethylsulfonyl fluoride and 0.1 mM sodium orthovanadate. The cells were lysed by flash freezing in a dry ice–methanol bath followed by rapid thawing at 37°C and Dounce homogenization. Insoluble material was removed by centrifugation as described above. The samples were adjusted to contain final concentrations of 1%

Triton X-100 and 1 mM DTT, and then were pre-cleared for 1 h at 4°C with 25 µl of protein A–Sepharose (Sigma) coupled to 5 µg of polyclonal rabbit anti-mouse IgG antibodies (Pierce). The cleared extracts were incubated for 30 min on ice with 3 µg of 12CA5 antibody, and the antigen–antibody complexes were mixed with 10 µl of protein A–Sepharose beads for 30 min at 4°C. The protein A–Sepharose beads were pre-blocked in 25 mM Tris, 150 mM NaCl supplemented with 0.5% (w/v) Triton X-100 and 5% powdered non-fat dry milk. The precipitated immune complexes were washed four times with homogenization buffer supplemented with 1% Triton X-100 and 1 mM DTT, once with high salt buffer (50 mM Tris–HCl, pH 7.4, 2 M LiCl and 1 mM DTT), and twice with kinase buffer. Where indicated, the beads were resuspended in kinase buffer and treated with the indicated concentration of wortmannin for 1 h at 24°C. The samples were then washed once with kinase buffer supplemented with 1 mM DTT. Kinase reactions, SDS–PAGE and autoradiography were performed as described above.

To determine the effect of wortmannin on recombinant mTOR autokinase activity in intact cells, Sf9 cells were infected in 100 mm tissue culture dishes as described above. After 36 h, the medium was replaced with Grace's medium (Gibco BRL), and the cells were treated with the indicated concentrations of wortmannin for 1 h at 27°C. The cells were lysed by scraping into homogenization buffer supplemented with 15 mM β-glycerophosphate, 250 mM NaCl, 2 mM EDTA, 1% Triton X-100 and the protease inhibitors as described above. The kinase activity of immunoprecipitated mTOR was also assayed as described in the preceding section.

[^{3}H]Wortmannin binding assays

Rat brain extract (1 mg protein/sample) was incubated with 200 nM 17-β-hydroxy-[^{3}H]wortmannin (sp. act. 6330 mCi/mmol) for 30 min at 24°C. The radiolabeled wortmannin was a kind gift of Dr Garth Powis (Arizona Cancer Center, Tucson, AR). Where indicated, extracts were pre-incubated with cold wortmannin, or with ATPγS or GTPγS as indicated, for 30 min prior to addition of [^{3}H]wortmannin. The wortmannin-bound mTOR was purified by precipitation with the GST–FKBP12·rapamycin affinity matrix as described above. The eluted proteins were separated by SDS–PAGE through a 7.5% gel, and radiolabeled proteins were detected by fluorography at –70°C after treating the gels with EN^{3}HANCE (DuPont NEN).

Acknowledgements

We thank the members of the laboratory for critical discussions and comments on the manuscript. We are grateful to Dr L.Karnitz for assistance with the preparation of the epitope-tagged mTOR cDNA construct, and to Dr Garth Powis for the generous supply of radiolabeled wortmannin. This work was supported by grants CA52995 (R.T.A.), DK28312 (J.C.L.) and AR41180 (J.C.L.) from the National Institutes of Health, by grant IM-19 (R.T.A.) from the American Cancer Society, and by the Mayo Foundation. R.T.A. is a Leukemia Society of America Scholar.

References

Abraham,R.T. (1996) Phosphatidylinositol 3-kinase related kinases. *Curr. Opin. Immunol.*, **8**, 412–418.

Abraham,R.T. and Wiederrecht,G.J. (1996) Immunopharmacology of rapamycin. *Annu. Rev. Immunol.*, **14**, 483–510.

Abraham,R.T., Ho,S.N. and McKean,D.J. (1987) Bioassay of interleukins. *J. Tissue Culture Methods*, **10**, 93–99.

Barbet,N.C., Schneider,U., Helliwell,S.B., Stansfield,I., Tuite,M.F. and Hall,M.N. (1996) TOR controls translation initiation and early G1 progression in yeast. *Mol. Biol. Cell*, **7**, 25–42.

Beretta,L., Gingras,A.-C., Svitkin,Y.V., Hall,M.N. and Sonenberg,N. (1996) Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J.*, **15**, 658–664.

Brown,E.J., Albers,M.W., Shin,T.B., Ichikawa,K., Keith,C.T., Lane,W.S. and Schreiber,S.L. (1994) A mammalian protein targeted by G1-arresting rapamycin–receptor complex. *Nature*, **369**, 756–758.

Brown,E.J., Beal,P.A., Keith,C.T., Chen,J., Shin,T.B. and Schreiber,S.L. (1995) Control of p70 S6 kinase by kinase activity of FRAP *in vivo*. *Nature*, **377**, 442–446.

Cafferkey,R. *et al.* (1993) Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. *Mol. Cell. Biol.*, **13**, 6012–6023.

Calvo,V., Crews,C., Vik,T. and Bierer,B. (1992) Interleukin 2 stimulation of p70 S6 kinase activity is inhibited by the immunosuppressant rapamycin. *Proc. Natl Acad. Sci. USA*, **89**, 7571–7575.

Cardenas,M.E. and Heitman,J. (1995) FKBP12–rapamycin target TOR2 in a vacuolar protein with an associated phosphatidylinositol-4 kinase activity. *EMBO J.*, **14**, 5892–5907.

Cheatham,B., Vlahos,C.J., Cheatham,L., Wang,L., Blenis,J. and Kahn,C.R. (1994) Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol. Cell. Biol.*, **14**, 4902–4911.

Chiu,M.I., Katz,H. and Berlin,V. (1994) RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. *Proc. Natl Acad. Sci. USA*, **91**, 12574–12578.

Chung,J., Kuo,C., Crabtree,G. and Blenis,J. (1992) Rapamycin–FKBP specifically blocks growth-dependent activation of and signaling by the 70 kDa S6 protein kinases. *Cell*, **69**, 1227–1236.

Dhand,R. *et al.* (1994) PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. *EMBO J.*, **13**, 522–533.

Ding,J., Vlahos,C.J., Liu,R., Brown,R.F. and Badwey,J.A. (1995) Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils. *J. Biol. Chem.*, **270**, 11684–11691.

Downward,J. (1994) Regulating S6 kinase. *Nature*, **371**, 378–379.

Dumont,F., Staruch,M., Koprak,S., Melino,M. and Sigal,N. (1990) Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. *J. Immunol.*, **144**, 251–258.

Dumont,F.J., Altmeyer,A., Kastner,C., Fischer,P.A., Lemon,K.P., Chung,J., Blenis,J. and Staruch,M.J. (1994) Relationship between multiple biologic effects of rapamycin and the inhibition of pp70S6 protein kinase activity. Analysis in mutant clones of a T cell lymphoma. *J. Immunol.*, **152**, 992–1003.

Dumont,F.J., Staruch,M.J., Grammer,T., Blenis,J., Kastner,C.A. and Rupprecht,K.M. (1995) Dominant mutations confer resistance to the immunosuppressant, rapamycin, in variants of a T cell lymphoma. *Cell. Immunol.*, **163**, 70–79.

Ferby,I.M., Waga,I., Sakanaka,C., Kume,K. and Shimizu,T. (1994) Wortmannin inhibits mitogen-activated protein kinase activation induced by platelet-activating factor in guinea pig neutrophils. *J. Biol. Chem.*, **269**, 30485–30488.

Graves,L.M., Bornfeldt,K.E., Argast,G.M., Krebs,E.G., Kong,X., Lin,T.A. and Lawrence,J.C.,Jr (1995) cAMP- and rapamycin-sensitive regulation of the association of eukaryotic initiation factor 4E and the translational regulator PHAS-I in aortic smooth muscle cells. *Proc. Natl Acad. Sci. USA*, **92**, 7222–7226.

Griffith,J.P. *et al.* (1995) X-ray structure of calcineurin inhibited by the immunophilin–immunosuppressant FKBP12–FK506 complex. *Cell*, **82**, 507–522.

Haghighat,A., Mader,S., Pause,A. and Sonenberg,N. (1995) Repression of cap-dependent translation initiation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. *EMBO J.*, **14**, 5701–5709.

Hall,M.N. (1996) The TOR signalling pathway and growth control in yeast. *Biochem. Soc. Trans.*, **24**, 234–239.

Han,J.-W., Pearson,R.B., Dennis,P.B. and Thomas,G. (1995) Rapamycin, wortmannin, and the methylxanthine SQ20006 inactivate p70^{s6k} by inducing dephosphorylation of the same subset of sites. *J. Biol. Chem.*, **36**, 21396–21403.

Hartley,K.O. *et al.* (1995) DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell*, **82**, 849–856.

Heitman,J., Movva,N.R. and Hall,M.N. (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science*, **253**, 905–909.

Helliwell,S.B., Wagner,P., Kunz,J., Deuter-Reinhard,M., Henriquez,R. and Hall,M.N. (1994) TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol. Biol. Cell*, **5**, 105–118.

Herbert,J.M., Seban,E. and Maffrand,J.P. (1990) Characterization of specific binding sites for [^{3}H]-staurosporine on various protein kinases. *Biochem. Biophys. Res. Commun.*, **171**, 189–195.

Hershey,J.W.B. (1991) Translational control in mammalian cells. *Annu. Rev. Biochem.*, **60**, 717–755.

Hunter,T. (1995) When is a lipid kinase not a lipid kinase? When it is a protein kinase. *Cell*, **83**, 1–4.

Kamps,M.P. and Sefton,B.M. (1989) Acid and base hydrolysis of phosphoproteins bound to immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. *Anal. Biochem.*, **176**, 22–27.

Karnitz,L.M., Burns,L.A., Sutor,S.L., Blenis,J. and Abraham,R.T. (1995) Interleukin-2 triggers a novel phosphatidylinositol 3-kinase-dependent MEK activation pathway. *Mol. Cell. Biol.*, **15**, 3049–3057.

Keith,C.T. and Schreiber,S.L. (1995) PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints. *Science*, **270**, 50–51.

King,L.A. and Possee,R.D. (1992) *The Baculovirus Expression System. A Laboratory Guide*. Chapman & Hall, London, UK.

Kunz,J., Henriquez,R., Schneider,U., Deuter-Reinhard,M., Movva,R. and Hall,M.N. (1993) Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G_1 progression. *Cell*, **73**, 585–596.

Kuo,C., Chung,J., Fiorentino,D., Flanagan,W., Blenis,J. and Crabtree,G. (1992) Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. *Nature*, **358**, 70–73.

Lin,T.-A., Kong,X., Haystead,T.A.J., Pause,A., Belsham,G., Sonenberg,N. and Lawrence,J.C.,Jr (1994) PHAS-I as a link between mitogen-activated protein kinase and translation initiation. *Science*, **266**, 653–656.

Lin,T.-A., Kong,X., Saltiel,A.R., Blackshear,P.J. and Lawrence,J.C.,Jr (1995) Control of PHAS-I by insulin in 3T3-L1 adipocytes. *J. Biol. Chem.*, **270**, 18531–18538.

Mader,S., Lee,H., Pause,A. and Sonenberg,N. (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4γ and the translational repressors 4E-binding proteins. *Mol. Cell. Biol.*, **15**, 4990–4997.

Monfar,M., Lemon,K.P., Grammer,T.C., Cheatham,L., Chung,J., Vlahos,C.J. and Blenis,J. (1995) Activation of pp70/85 S6 kinases in interleukin-2-responsive lymphoid cells is mediated by phosphatidylinositol 3-kinase and inhibited by cyclic AMP. *Mol. Cell. Biol.*, **15**, 326–337.

Morice,W.G., Wiederrecht,G., Brunn,G.J., Siekierka,J.J. and Abraham, R.T. (1993) Rapamycin inhibition of interleukin-2-dependent $p33^{cdk2}$ and $p34^{cdc2}$ kinase activation in T lymphocytes. *J. Biol. Chem.*, **268**, 22737–22745.

Nourse,J., Firpo,E., Flanagan,W.M., Coats,S., Polyak,K., Lee,M.-H., Massague,J., Crabtree,G.R. and Roberts,J.M. (1994) Interleukin-2-mediated elimination of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature*, **372**, 570–573.

Pause,A., Belsham,G.J., Gingras,A.-C., Donz,O., Lin,T.-A., Lawrence, J.C.,Jr and Sonenberg,N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. *Nature*, **371**, 762–767.

Powis,G. *et al.* (1994) Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Res.*, **54**, 2419–2423.

Price,D., Grove,J., Calvo,V., Avruch,J. and Bierer,B. (1992) Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. *Science*, **257**, 973–977.

Ruegg,U.T. and Burgess,G.M. (1989) Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends Pharmacol. Sci.*, **10**, 218–220.

Sabatini,D.M., Erdjument-Bromage,H., Lui,M., Tempst,P. and Snyder, S.H. (1994) RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell*, **78**, 35–43.

Sabatini,D.M., Pierchala,B.A., Barrow,R.K., Schell,M.J. and Snyder,S.H. (1995) The rapamycin and FKBP12 target (RAFT) displays phosphatidylinositol 4-kinase activity. *J. Biol. Chem.*, **270**, 20875–20878.

Sabers,C.J., Martin,M.M., Brunn,G.J., Williams,J.M., Dumont,F.J., Wiederrecht,G. and Abraham,R.T. (1995) Isolation of a protein target of the FKBP12–rapamycin complex in mammalian cells. *J. Biol. Chem.*, **270**, 815–822.

Skorski,T., Kanakaraj,P., Nieborowska-Skorska,M., Ratajczak,M.Z., Wen,S.-C., Zon,G., Gewirtz,A.M., Perussia,B. and Calabretta,B. (1995) Phosphatidylinositol-3 kinase activity is regulated by BCR/ABL and is required for the growth of Philadelphia chromosome-positive cells. *Blood*, **86**, 726–736.

Sonenberg,N. (1993) Remarks on the mechanism of ribosome binding to eukaryotic mRNAs. *Gene Expression*, **3**, 317–323.

Stack,J.H. and Emr,S.D. (1994) Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific PI 3-kinase activities. *J. Biol. Chem.*, **269**, 31552–31562.

Terada,N., Lucas,J.J., Szepesi,A., Franklin,R.A., Domenico,J. and Gelfand,E.W. (1993) Rapamycin blocks cell cycle progression of activated T cells prior to events characteristic of the middle to late G_1 phase of the cycle. *J. Cell. Physiol.*, **154**, 7–15.

Ui,M., Okada,T., Hazeki,K. and Hazeki,O. (1995) Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem. Sci.*, **20**, 303–307.

Vlahos,C.J., Matter,W.F., Hui,K.Y. and Brown,R.F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.*, **269**, 5241–5248.

Wymann,M.P., Bulgarelli-Leva,G., Zvelebil,M.J., Pirola,L., Vanhaesebroeck,B., Waterfield,M.D. and Panayotou,G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.*, **16**, 1722–1733.

von Manteuffel,S.R., Gingras,A.-C., Ming,X.-F., Sonenburg,N. and Thomas,G. (1996) 4E-BP1 phosphorylation is mediated by the FRAP–$p70^{s6k}$ pathway and is independent of mitogen-activated protein kinase. *Proc. Natl Acad. Sci. USA*, **93**, 4076–4080.

Zakian,V.A. (1995) ATM-related genes: what do they tell us about functions of the human gene? *Cell*, **82**, 685–687.

Zheng,X.-F., Fiorentino,D., Chen,J., Crabtree,G.R. and Schreiber,S.L. (1995) TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin. *Cell*, **82**, 121–130.

Received on April 29, 1996; revised on June 13, 1996