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The Rapamycin and FKBP12 Target (RAFT) Displays Phosphatidylinositol 4-Kinase Activity*

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The immunosuppressant rapamycin prevents cell cycle progression in several mammalian cell lines and the yeast *Saccharomyces cerevisiae*. In mammalian cells, rapamycin binds to the small FK506-binding protein, FKBP12, allowing the drug-receptor complex to interact with the 289-kDa RAFT1/FRAP proteins. These proteins, along with their yeast homologs, TOR1/DRR1 and TOR2/DRR2, contain a C-terminal domain with amino acid homology to several phosphatidylinositol (PI) 4- and 3-kinases. However, no direct demonstration of kinase activity for this family of proteins has been reported. We now show that RAFT1, immunoprecipitated from rat brain and MG63 and HEK293 cells, contains PI 4-kinase activity and that rapamycin-FKBP12 has no effect on this activity. Thus, it is likely that, *in vivo*, rapamycin does not directly inhibit the PI 4-kinase activity and affects the RAFT1/FRAP protein through another mechanism.

The ability of rapamycin, a potent immunosuppressant drug, to arrest a variety of mammalian cells and the yeast *Saccharomyces cerevisiae* in the G_1 stage of the cell cycle (1–4) has made it a valuable tool for studying the intermediate signaling events that convey mitogenic stimuli to the cell nucleus.

Rapamycin binds with low nanomolar affinity to the FK506-binding protein (FKBP12), a small soluble protein that is also the intracellular receptor for FK506, a structurally similar immunosuppressant (5–7). Analogous to the mechanism of action of FK506, it is the drug-receptor complex that mediates the effects of the rapamycin, which include inhibition of the 70-kDa S6 kinase (8, 9) and of several cyclin-dependent kinases (2–4). The immunosuppressant effects of FK506 derive from the binding of the FK506-FKBP12 complex to the calcium-activated phosphatase, calcineurin, and the resulting inhibition of its activity (10). Although rapamycin binds to FKBP12, the complex does not interact with calcineurin. Instead, rapamycin-FKBP12 binds to a recently purified and molecularly cloned protein designated rapamycin and FKBP12 target-1 (RAFT1) in rats (11) and FKBP-rapamycin-associated protein (FRAP) in humans (12). Several other groups have subsequently purified and/or cloned the same protein (13–15).

RAFT1 is a 2549-amino acid protein with a predicted molecular mass of 289 kDa and is thought to be a mammalian homolog of the products of two yeast genes, *TOR1/DRR1* and *TOR2/DRR2*, which when mutated lead to dominant rapamycin resistance in yeast (1, 16, 17). The C-terminal 600-amino acid domain of RAFT1 and the yeast TOR proteins has homology to several PI[1] kinases, including the p110 subunit of mammalian PI 3-kinase (18); the yeast PI 3-kinase VPS34 (19); two yeast PI 4-kinases, STT4 (20) and PIK1 (21, 22); and the recently cloned mammalian PI 4-kinase, PI4Kα (23). This homology to known lipid kinases has lead to the suggestion that RAFT1 may also be a PI kinase and that rapamycin exerts its effects by inhibiting this activity (11). However, no direct demonstration of kinase activity for RAFT1/FRAP or the yeast TOR proteins has been reported.

In this study, we show that immunoprecipitates of endogenous RAFT1 from rat brain or tissue culture cells contain PI 4-kinase activity and that, surprisingly, rapamycin-FKBP12 has no effect on this activity.

EXPERIMENTAL PROCEDURES

Materials—Materials were purchased from the following sources: PI, PI-4-P, PI-4,5-P_2, wortmannin, and FKBP12 from Sigma; PS from Avanti Polar Lipids, Inc. (Alabaster, AL); [γ-^{32}P]ATP (AA0018) from Amersham Corp.; [^{3}H]PI-4-P and [^{3}H]PI-4,5-P_2 from American Radiolabeled Chemicals; silica plates from E. Merck AG (Darmstadt, Germany); protein A-agarose from Oncogene Science Inc. (Uniondale, NY); and horseradish peroxidase-conjugated goat anti-rabbit antibody from Boehringer Mannheim. Rapamycin was a generous gift of the Wyeth-Ayerst Co. (Philadelphia, PA), and FK506 was a gift of Fujisawa Co. (Tsukuba City, Japan). Three peptides corresponding to amino acids 64–79 (peptide 64), 782–797 (peptide 782), and 2528–2549 (peptide C-term) were synthesized, coupled to BSA, and used in the generation of rabbit polyclonal antibodies, which were affinity-purified before use for Western blotting or immunoprecipitation (24, 25). MG63 and HEK293 cells were grown as described (26).

Immunoprecipitation and Immunoblotting—Fresh rat brains were homogenized in 6 ml/brain of 20 mM Hepes (pH 6.8), 150 mM KCl, 1 mM EGTA, 0.1 mM EDTA, 50 mM NaF, 1.5 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml pepstatin, and 0.5 μg/ml leupeptin (HKEE buffer). The homogenate was centrifuged at 35,000 $\times$ *g* for 30 min, and to the supernatant, additions were made to attain final concentrations of 1 mM $MgCl_2$, 0.05% SDS, and 0.1% CHAPS. The supernatant was precleared for 40 min with 40 μl of protein-agarose/ml and immunoprecipitated for 2–3 h at 4 °C with 8 μg/ml antibody alone or antibody that had been preincubated with 50 μg of blocking peptide. The immunocomplexes were recovered with 15 μl of protein A-agarose and washed twice with HKEE buffer containing 1% Nonidet P-40, twice with HKEE buffer containing 1% Nonidet P-40 and 0.5 M LiCl, twice with HKEE buffer, and once with 50 mM Hepes (pH 7.4) containing 120 mM NaCl. Immunoprecipitations from MG63 and HEK293 cells were done as for brain except that 1 ml/10-cm dish of HKEE buffer with $MgCl_2$, SDS, and CHAPS was used to lyse the cells, and the insoluble material was

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[1] The abbreviations used are: PI, phosphatidylinositol; PS, phosphatidylserine; PIP, phosphatidylinositol phosphate; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography.

removed by centrifugation for 15 min at 15,000 × *g* in a microcentrifuge. HEK293 cells transfected by the $CaPO_4$ method with RAFT1 or vector alone were lysed in the same buffer.

Proteins separated by 6% SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose on a semidry apparatus using Dunn carbonate buffer (27). Blots were blocked for 1 h in PBS with 5% milk, incubated overnight with 2 μg/ml antibody in PBS with 3% BSA, washed 3 × 5 min in PBS with 5% milk, incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit secondary antibody, washed as before, and detected with ECL (Amersham Corp.).

PI Kinase Assay and HPLC Analysis—Immunoprecipitates were incubated for 10 min on ice in 40 μl of 30 mM Hepes (pH 7.4) containing 0.5 mg/ml PS, 0.25 mg/ml PI, 1 mM dithiothreitol, and 0.2% Nonidet P-40. When used, PI-4-P or PI-4,5-P_2 was substituted for PI at 0.25 mg/ml. The kinase reaction was started by firing in 20 μCi of [γ-^{32}P]ATP in 10 μl of a 5 × concentrate consisting of 150 mM Hepes (pH 7.4), 40 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mg/ml fatty acid-free BSA, 5 mM dithiothreitol, and 250 μM ATP. The reaction was continued for 20 min at room temperature and quenched with 100 μl of 1 M HCl, and the phosphorylated lipids were extracted and separated by TLC as described (28). The reaction was linear up to 30 min. Phosphorylated lipids were excised from the TLC plate, deacylated (29), and analyzed by strong anion exchange HPLC (28). To determine if the product of kinase activity and the standard precisely coelute from the HPLC column, we collected eight fractions/min during the 20–35-min period of the HPLC run.

RESULTS AND DISCUSSION

Characterization of Anti-RAFT1 Antibodies—In initial experiments, we attempted to demonstrate kinase activity of a recombinant RAFT1 protein overexpressed in HEK293 cells. We immunoprecipitated the RAFT1 protein epitope-tagged at its N terminus and evaluated the kinase activity of the immunoprecipitates toward a wide variety of lipids, including phosphatidylinositols, sphingolipids, ceramide, and traditional protein substrates such as caseins and histones. We failed to detect kinase activity toward any of these substrates (data not shown).

The inability to detect kinase activity of recombinant RAFT1 might reflect an improper folding of the overexpressed protein, an interference by the epitope tag, or the absence of post-translational modifications or of another subunit necessary for activity. To investigate the lipid kinase activity of endogenous RAFT1, we developed antibodies to distinct portions of the RAFT1 protein itself, using as immunogens three synthetic peptides corresponding to regions at the N and C termini and within the central portion of RAFT1 (Fig. 1*A*). The α-64-RAFT1 antibody recognizes a single band with an apparent molecular mass of 220 kDa in extracts from brain and MG63 osteosarcoma cells as well as from control HEK293 cells or HEK293 cells that overexpress RAFT1 (Fig. 1*B*). Similarly, antibodies α-782-RAFT1 and α-C-term-RAFT1 recognize a protein of the same apparent molecular mass (data not shown), which also corresponds to that of the RAFT1 protein purified using a rapamycin-FKBP12 affinity column (11–13, 15).

We conducted immunoprecipitation experiments in combination with Western blotting to ascertain that the epitopes recognized by the three different antibodies are all part of the same molecule. When α-64-RAFT1 is used to probe Western blots of immunoprecipitates prepared with each of the three anti-RAFT1 antibodies, a band of M_r = 220,000 is always detected, which is eliminated by preincubation of the immunoprecipitating antibody with the peptide against which it was raised (Fig. 1*C*). Immunoreactive bands of lower molecular weight that are also eliminated by antigen preincubation probably represent degradation products of the large RAFT1 protein.

RAFT1 Immunoprecipitates Exhibit PI 4-Kinase Activity—RAFT1 immunoprecipitates prepared from detergent-solubilized membranes from brain and MG63 and HEK293 cells were assayed for lipid kinase activity using thin-layer chromatography to analyze the phosphorylated lipids. Using PI as a substrate, PIP is produced in RAFT1 immunoprecipitates obtained from all three sources (Fig. 2*A*) using two different α-RAFT1 antibodies. No phosphorylated product is seen when PI-4-P or PI-4,5-P_2 is used as a substrate instead of PI (data not shown). The PI kinase activity is completely blocked in the brain membrane extract and to a major extent in the cell culture preparations when the antibodies are preincubated with blocking peptides prior to use for immunoprecipitation. It is possible, however, that the PI kinase activity of RAFT1 immunoprecipitates is due to a RAFT1-associated protein that coimmunoprecipitates with RAFT1.

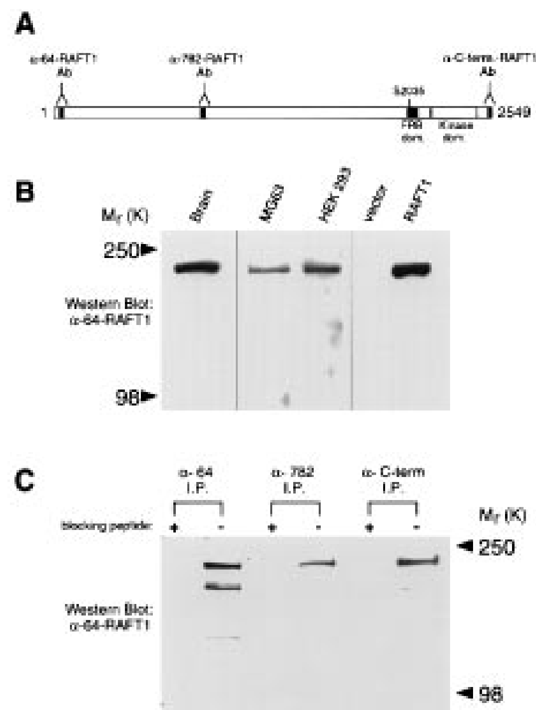

FIG. 1. **Characterization of anti-RAFT1 peptide antibodies.** *A*, shown is a diagram of the location of the RAFT1 sequences that were used in the generation of peptide antibodies (*Ab*), the minimal FKBP-rapamycin-binding domain (*FRB*) (47), the serine (Ser-2035) that when mutated to arginine inhibits FKBP-rapamycin binding (47), and the putative lipid kinase domain. *B*, on Western blots, the α-64-RAFT1 antibody recognizes a 220-kDa protein in membranes from rat brain (60 μg), MG63 cells (150 μg), and HEK293 cells (150 μg) and in detergent lysates (10 μg) of 293 cells transfected with a cytomegalovirus vector containing the cDNA for RAFT1 (*RAFT1*), but not with the vector alone (*vector*). The ECL exposure times were as follows: brain, 1 min; MG63 and 293 cells, 2 min; and transfected cells, 30 s. *C*, the α-64-RAFT1 antibody immunoprecipitates a 220-kDa protein and recognizes proteins of the same molecular mass in immunoprecipitates made with two other α-RAFT1 peptide antibodies. Shown is a Western blot probed with the α-64-RAFT1 antibody of immunoprecipitates (*I.P.*) prepared from rat brain with the α-64-RAFT1, α-782-RAFT1, and α-C-term-RAFT1 antibodies (−) or with the antibodies preincubated with the peptides used in their generation (+). The ECL exposure time was 30 s.

To characterize the type of PI kinase involved, we took advantage of the known properties of certain PI kinases (30). Type II PI 4-kinase is activated by nonionic detergents and inhibited by adenosine, while type III PI 4-kinase activity is much less sensitive to either treatment. The PI kinase activity is stimulated over 20-fold by the detergent Nonidet P-40, but is decreased only ~20% with a high concentration of adenosine (Fig. 2*B*), indicating that it does not represent either a conventional type II or III PI 4-kinase.

Wortmannin is a potent *in vitro* inhibitor of PI 3-kinase that is used extensively to implicate this enzyme in biological processes (31). The observation that both wortmannin and rapamycin inhibit p70 S6 kinase activity (32) raises the possibility

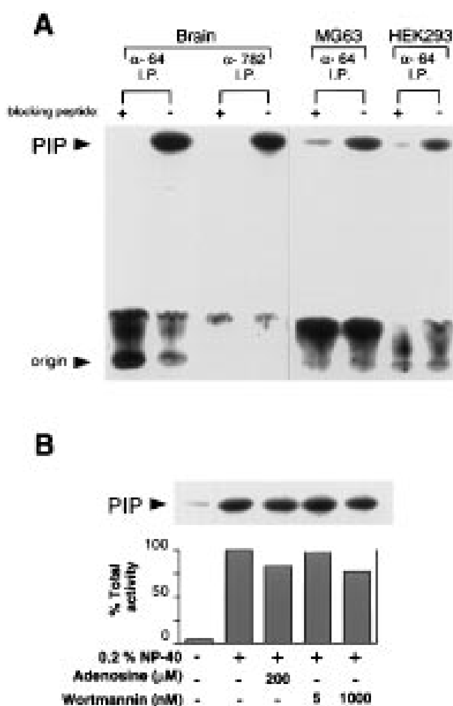

FIG. 2. **RAFT1 immunoprecipitates contain PI kinase activity that is activated by nonionic detergent and not inhibited by adenosine or wortmannin.** *A*, PI kinase assays using PI and PS as substrates in the presence of 0.2% Nonidet P-40 were performed on RAFT1 immunoprecipitates (*I.P.*) from brain or MG63 or HEK293 cells prepared as described for Fig. 1*B*. Phosphorylated lipids were chloroform-extracted, separated by TLC, and detected by autoradiography. The migration position of the PIP standard is indicated. The TLC plate was exposed to film for 10.5 h (brain), 36 h (MG63), and 24 h (HEK293). *B*, RAFT1 was immunoprecipitated from brain with the α-64-RAFT1 antibody and assayed for PI kinase activity under the conditions indicated. Shown is an autoradiograph of the TLC plate (*top panel*). The activity of each reaction was quantitated by densitometry of the autoradiograph and is presented as percentage of total activity in the presence of 0.2% Nonidet P-40 (*bottom panel*). This experiment was repeated three times with similar results.

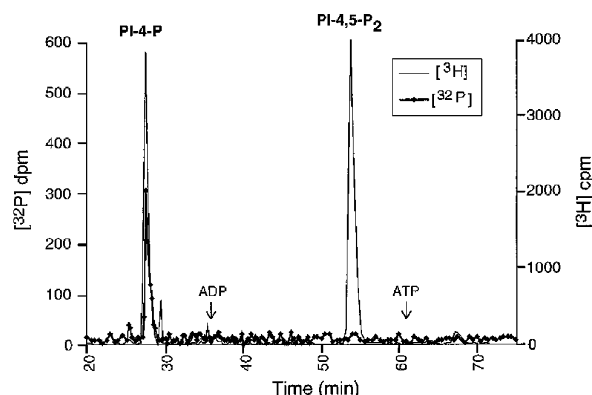

FIG. 3. **The PIP generated by RAFT1 immunoprecipitates is PI-4-P.** The ^{32}P-labeled PIP generated as described for Fig. 2*A* was excised from the TLC plate, deacylated (29), and subjected to HPLC analysis (28). The positions of the standards [^{3}H]PI-4-P and [^{3}H]PI-4,5-P_2 are shown (*thin line*). The [^{32}P]PIP (*thick line* with *diamonds*) comigrates with the PI-4-P standard. The positions of ADP and ATP, internal standards used to compare separate HPLC analyses, are indicated with *arrows*. This experiment was repeated three times with similar results. In addition, HPLC analyses demonstrate that the PIP generated by the RAFT1 immunoprecipitates in the absence of detergent is also PI-4-P (data not shown).

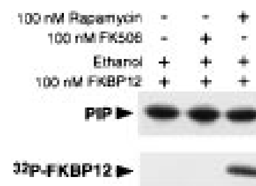

FIG. 4. **Rapamycin-FKBP12 does not inhibit the RAFT1 PI 4-kinase activity, but is able to bind to RAFT1 immunoprecipitates.** *Top panel*, RAFT1 immunoprecipitates were preincubated with 100 nM FKBP12, 100 nM FK506-FKBP12, or 100 nM rapamycin-FKBP12, and PI kinase assays were performed. All samples contain equivalent amounts of ethanol, the vehicle for the drugs. The experiment was repeated five times with similar results. *Bottom panel*, RAFT1 immunoprecipitates were treated as described above, except that 10,000 cpm of ^{32}P-labeled FKBP12 (11) were substituted for unlabeled FKBP12. After a 45-min incubation, the immunoprecipitates were washed twice and resolved by 18% SDS-polyacrylamide gel electrophoresis, and the dried gel was exposed to film. The experiment was repeated twice with similar results.

that these drugs target the same molecule, *i.e.* RAFT1. We detect no inhibition of PI kinase activity with 5 nM wortmannin, a concentration sufficient to inhibit the known mammalian PI 3-kinase (31), and only a slight reduction in PI kinase activity at 1000 nM wortmannin (Fig. 2*B*), a concentration that inhibits reported PI 3-kinase activities (33, 34), type III PI 4-kinase activity (34), and a novel wortmannin-sensitive PI 4-kinase activity (35). The inability of wortmannin to inhibit RAFT1 PI kinase activity indicates that RAFT1 is not likely to be a target of wortmannin *in vivo* and that the similar effects of wortmannin and rapamycin upon p70 S6 kinase activity are mediated through different molecules. Recent studies that identify the domains of the p70 S6 kinase molecule required for its mitogen-stimulated activation and rapamycin and wortmannin inhibition support this conclusion (36).

To identify the position on the inositol ring of PI that is phosphorylated by RAFT1 immunoprecipitates, we conducted HPLC analysis on the PIP produced after its deacylation (Fig. 3). The ^{32}P-labeled product of the phosphorylation activity migrates identically to the [^{3}H]PI-4-P standard. In this HPLC system, PI-3-P reproducibly elutes from the column at an earlier time point (28).

Rapamycin Does Not Inhibit the PI 4-Kinase Activity of RAFT1 Immunoprecipitates—The immunosuppressant actions of FK506 derive from the binding of the FK506-FKBP12 complex to calcineurin and subsequent inhibition of calcineurin phosphatase activity (10). Analogously, one might expect that the binding of the rapamycin-FKBP12 complex to RAFT1 inhibits the PI 4-kinase activity. Accordingly, we evaluated the effect of rapamycin, in the presence of FKBP12, upon RAFT1 kinase activity. Rapamycin, like the FK506 control, has no effect upon the RAFT1 PI 4-kinase activity of brain tissue (Fig. 4, *top panel*). The concentration of rapamycin employed, 100 nM, is 5–10-fold more than is necessary to produce the *in vivo* effects of the drug, such as the inhibition of p70 S6 kinase. In other experiments, rapamycin concentrations ranging from 10 nM to 1 μM also do not affect the lipid kinase activity (data not shown). Similar results are obtained with the MG63 and HEK293 cell lines (data not shown).

The failure of rapamycin-FKBP12 to inhibit the PI 4-kinase activity in the previous experiments could result from an inability of the drug-receptor complex to interact with RAFT1 after it has been immunoprecipitated. We showed, however, that under the experimental conditions used in the lipid kinase assay, ^{32}P-labeled FKBP12 does bind to the immunoprecipitated RAFT1 in a rapamycin-dependent fashion (Fig. 4, *bottom panel*). Furthermore, the addition of rapamycin to brain homogenates prior to immunoprecipitation in order to elicit the binding of rapamycin-FKBP12 to RAFT1 also does not result in an inhibition of the PI 4-kinase activity of the immunoprecipi-

tates (data not shown).

In summary, we have demonstrated that immunoprecipitates of RAFT1, which has a lipid kinase domain with amino acid homology to both PI 4- and 3-kinases, contain PI 4-kinase activity. We have only tested PI, PI-4-P, and PI-4,5-P_2 as *in vitro* substrates, and it is possible that RAFT1 could act upon other phosphatidylinositols that already possess more than one phosphate. For instance, RAFT1 might phosphorylate PI-3-P on the 4-position, and it may be the true *in vivo* substrate. Furthermore, our results do not rule out that RAFT1 may phosphorylate specific protein substrates. The mammalian PI 3-kinase and the yeast PI 3-kinase VPS34 display protein kinase activity (37–39), but this activity has not been reported for PI 4-kinases.

The inability of rapamycin-FKBP12 to inhibit the PI 4-kinase activity of RAFT1 raises questions about the mechanism of rapamycin's pharmacological actions. Although our results do not address the effects of rapamycin-FKBP12 on the PI 4-kinase activity of RAFT1 *in vivo*, it is likely that the drug does not directly inhibit the kinase activity of the protein. The drug could still effectively inhibit the lipid kinase activity by altering the intracellular localization of the enzyme and preventing its access to lipid substrates located on membranes. In addition, rapamycin-FKBP12 may prevent RAFT1 from interacting with substrates for a putative protein kinase activity or inhibit post-translational modifications of RAFT1, such as phosphorylation, that may be necessary for activity. Alternatively, RAFT1 may associate with a PI 4-kinase that coimmunoprecipitates with RAFT1 but is unaffected by rapamycin-FKBP12.

The finding that RAFT1 immunoprecipitates contain PI 4-kinase activity is also of importance outside of the field of immunophilin research. Multiply phosphorylated forms of PI are involved in numerous aspects of signal transduction. Most notably, PI-4-P is the precursor for PI-4,5-P_2, which is hydrolyzed by phospholipase C into inositol 1,4,5-trisphosphate and diacylglycerol, important mediators of intracellular calcium release and protein kinase C activity, respectively (40). PI-4-P synthesis can be activated by ligand binding to membrane receptors for neurotransmitters, hormones, and growth factors (41–43). Therefore, one might expect that the lipid kinase activity of RAFT1 will be tightly regulated.

PI-4-P and PI-4,5-P_2 are emerging as important regulators of the cytoskeleton and of vesicle fusion during membrane trafficking (44). Furthermore, the finding that the gene product mutated in ataxia telangiectasia (45) is a RAFT1 homolog indicates that derangements of phosphoinositide synthesis may cause human disease. The cDNA for only one PI 4-kinase has been isolated from mammalian tissues (23). The identification of these PI 4-kinases, as well as the recent cloning of the cDNA for a PI-4-P 5-kinase (46), may clarify the regulation of phosphoinositide biosynthesis and their role in controlling cell growth.

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REFERENCES

1. Heitman, J., Movva, N. R., and Hall, M. N. (1994) *Science* **253,** 905–909
2. Morice, W. G., Wiederrecht, G., Brunn, G. J., Siekierka, J. J., and Abraham, R. T. (1993) *J. Biol. Chem.* **268,** 22737–22745
3. Albers, M. W., Williams, R. T., Brown, E. J., Tanaka, A., Hall, F. L., and Schreiber, S. L. (1993) *J. Biol. Chem.* **268,** 22825–22829
4. Jayaraman, T., and Marks, A. R. (1993) *J. Biol. Chem.* **268,** 25385–25388
5. Bierer, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G., and Schreiber, S. L. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9231–9235
6. Dumont, F. J., Staruch, M. J., Koprak, S. L., Melino, M. R., and Sigal, N. H. (1990) *J. Immunol.* **144,** 251–258
7. Dumont, F. J., Melino, M. R., Staruch, M. J., Koprak, S. L., Fisher, P. A., and Sigal, N. H. (1990) *J. Immunol.* **144,** 1418–1424
8. Chung, J., Kuo, C. J., Crabtree, G. R., and Blenis, J. (1992) *Cell* **69,** 1227–1236
9. Kuo, C. J., Chung, J., Fiorentino, D. F., Flanagan, W. M., Blenis, J., and Crabtree, G. R. (1992) *Nature* **358,** 70–73
10. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) *Cell* **66,** 807–815
11. Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S. H. (1994) *Cell* **78,** 35–43
12. Brown, E. J., Albers, M. W., Shin, T. B., Ocjlawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994) *Nature* **369,** 756–758
13. Chen, Y., Chen, H., Rhoad, A. E., Warner, L., Caggiano, T. J., Failli, A., Zhang, H., Hsiao, C.-L., Nakanishi, K., and Molnar-Kimber, K. L. (1994) *Biochem. Biophys. Res. Commun.* **203,** 1–7
14. Chiu, M. I., Katz, H., and Berlin, V. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12574–12578
15. Sabers, C. J., Martin, M. M., Brunn, G. J., Williams, J. M., Dumont, F. J., Wiederrecht, G., and Abraham, R. T. (1995) *J. Biol. Chem.* **270,** 815–822
16. Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N. R., and Hall, M. N. (1993) *Cell* **73,** 585–596
17. Cafferkey, R., Young, P. R., McLaughlin, M. M., Bergsma, D. J., Koltin, Y., Sathe, G. M., Faucette, L., Eng, W. K., Johnson, R. K., and Livi, G. P. (1993) *Mol. Cell. Biol.* **13,** 6012–6023
18. Carpenter, C. L., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S., and Cantley, L. C. (1990) *J. Biol. Chem.* **265,** 19704–19711
19. Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., and Emr, S. D. (1993) *Science* **260,** 88–91
20. Yoshida, S., Ohya, Y., Goebl, M., Nakano, A., and Anaraku, Y. (1994) *J. Biol. Chem.* **269,** 1166–1172
21. Flanagan, C. A., Schnieders, E. S., Emerick, A. W., Kunisawa, R., Admon, A., and Thorner, J. (1993) *Science* **262,** 1444–1448
22. Garcia-Bustos, J. F., Marini, F., Stevenson, I., Frei, C., and Hall, M. N. (1994) *EMBO J.* **13,** 2352–2361
23. Wong, K., and Cantley, L. C. (1994) *J. Biol. Chem.* **269,** 28878–28884
24. Blackstone, C. D., Moss, S. J., Martin, L. J., Levey, A. I., Price, D. L., and Huganir, R. L. (1992) *J. Neurochem.* **58,** 1–9
25. Blackstone, C. D., Levey, A. I., Martin, L. J., Price, D. L., and Huganir, R. L. (1992) *Ann. Neurol.* **31,** 680–683
26. Carbonaro-Hall, D., Williams, R., Wu, L., Warburton, D., Zeichner-David, M., MacDougall, M., Tolo, V., and Hall, F. (1993) *Oncogene* **8,** 1649–1659
27. Dunn, S. D. (1986) *Anal. Biochem.* **157,** 144–153
28. Auger, K. R., Serunian, L. A., and Cantley, L. C. (1990) in *Methods in Inositide Research* (Irvine, R. F., ed) pp. 159–165, Raven Press, New York
29. Van Veldhoven, P. P., Matthews, T. J., Bolognesi, D. P., and Bell, R. M. (1992) *Biochem. Biophys. Res. Commun.* **187,** 209–216
30. Carpenter, C. L., and Cantley, L. C. (1990) *Biochemistry* **29,** 11147–11156
31. Thelen, M., Wymann, M. P., and Langen, H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4960–4964
32. Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994) *Nature* **370,** 71–75
33. Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweis, P. C., and Hawkins, P. T. (1994) *Cell* **77,** 83–93
34. Stephens, L., Cooke, F. T., Walters, R., Jackson, T., Volinia, S., Gout, I., Waterfield, M. D., and Hawkins, P. T. (1994) *Curr. Biol.* **4,** 203–214
35. Nakanishi, S., Catt, K. J., and Balla, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5317–5321
36. Weng, Q.-P., Andrabi, K., Kozlowski, M. T., Grove, J. R., and Avruch, J. (1995) *Mol. Cell. Biol.* **15,** 2333–2340
37. Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N. F., Truong, O., Vicendo, P., Yonezawa, K., Kasuga, M., Courtneidge, S. A., and Waterfield, M. D. (1994) *EMBO J.* **13,** 522–533
38. Lam, K., Carpenter, C. L., Ruderman, N. B., Friel, J. C., and Kelly, K. L. (1994) *J. Biol. Chem.* **269,** 20648–20652
39. Stack, J. H., and Emr, S. D. (1994) *J. Biol. Chem.* **269,** 31552–31562
40. Divecha, N., and Irvine, R. F. (1995) *Cell* **80,** 269–278
41. Walker, D. W., and Pike, L. J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7513–7517
42. Cochet, C., Filhol, O., Payrastre, B., Hunter, T., and Gill, G. N. (1991) *J. Biol. Chem.* **266,** 637–644
43. Payrastre, B., van Bergen en Henegouwen, P. M. P., Breton, M., den Hartigh, J. C., Plantavid, M., Verkleij, A. J., and Boonstra, J. (1991) *J. Cell Biol.* **115,** 121–128
44. Janmey, P. A. (1995) *Chem. & Biol.* **2,** 61–65
45. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S. R., Simmons, A., Clines, G. A., Sartiel, A., Gatti, R. A., Chessa, L., Sanal, O., Lavin, M. F., Jaspers, N. G. J., Malcolm, A., Taylor, R., Arlett, C. F., Miki, T., Weissman, S. M., Lovett, M., Collins, F. S., and Shiloh, Y. (1995) *Science* **268,** 1749–1753
46. Boronenkov, I. V., and Anderson, R. A. (1995) *J. Biol. Chem.* **270,** 2881–2884
47. Chen, J., Zheng, X.-F., Brown, E. J., and Schreiber, S. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4947–4951