MOLECULAR AND CELLULAR BIOLOGY, Jan. 1995, p. 326–337 Vol. 15, No. 1
0270-7306/95/$04.00+0

Activation of pp70/85 S6 Kinases in Interleukin-2-Responsive Lymphoid Cells Is Mediated by Phosphatidylinositol 3-Kinase and Inhibited by Cyclic AMP

MERCÈ MONFAR,[1] KATHERINE P. LEMON,[1]† TIMOTHY C. GRAMMER,[1] LYNN CHEATHAM,[1] JONGKYEONG CHUNG,[1] CHRIS J. VLAHOS,[2] AND JOHN BLENIS[1]*

Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115,[1] and Cardiovascular Research, Lilly Research Laboratories, Indianapolis, Indiana 46285[2]

Received 27 May 1994/Returned for modification 14 July 1994/Accepted 13 October 1994

Activation of phosphatidylinositol 3-kinase (PI3K) and activation of the 70/85-kDa S6 protein kinases (αII and αI isoforms, referred to collectively as pp70^{S6k}) have been independently linked to the regulation of cell proliferation. We demonstrate that these kinases lie on the same signalling pathway and that PI3K mediates the activation of pp70 by the cytokine interleukin-2 (IL-2). We also show that the activation of pp70^{S6k} can be blocked at different points along the signalling pathway by using specific inhibitors of T-cell proliferation. Inhibition of PI3K activity with structurally unrelated but highly specific PI3K inhibitors (wortmannin or LY294002) results in inhibition of IL-2-dependent but not phorbol ester (conventional protein kinase C [cPKC])-dependent pp70^{S6k} activation. The T-cell immunosuppressant rapamycin potently antagonizes IL-2- (PI3K)- and phorbol ester (cPKC)-mediated activation of pp70^{S6k}. Thus, wortmannin and rapamycin antagonize IL-2-mediated activation of pp70^{S6k} at distinct points along the PI3K-regulated signalling pathway, or rapamycin antagonizes another pathway required for pp70^{S6k} activity. Agents that raise the concentration of intracellular cyclic AMP (cAMP) and activate cAMP-dependent protein kinase (PKA) also inhibit IL-2-dependent activation of pp70^{S6k}. In this case, inhibition appears to occur at least two points in this signalling path. Like rapamycin, PKA appears to act downstream of cPKC-mediated pp70^{S6k} activation, and like wortmannin, PKA antagonizes IL-2-dependent activation of PI3K. The results with rapamycin and wortmannin are of added interest since the yeast and mammalian rapamycin targets resemble PI3K in the catalytic domain.

T cells are activated, representing G_0-to-G_1 transition, by antigen presentation to the multimeric T-cell receptor. This results in the transcription, production, and secretion of the 15-kDa glycoprotein lymphokine, interleukin-2 (IL-2). Antigen stimulation also induces expression of the IL-2 receptor (IL-2R) α subunit (p55) and increases the level of IL-2R β subunit (p75). Together with the γ subunit, they form the high-affinity IL-2R (reviewed in references 75 and 103). IL-2 then stimulates activated T cells in an autocrine/paracrine fashion, driving G_1-S transition and cell proliferation. The IL-2R has no intrinsic kinase activity, yet ligand binding increases tyrosine phosphorylation of many proteins, including the IL-2R β chain. IL-2-dependent signalling also results in activation of c-Ras and phosphatidylinositol 3-kinase (PI3K) and increased serine/threonine protein phosphorylation. Although much is known about Ras-regulated signal transduction (see references 12, 39, and 68 for reviews), the identities of the signalling proteins lying downstream of PI3K remain to be established.

PI3K is a novel signal transducer composed of an 85-kDa SH2- and SH3-domain-containing regulatory subunit and a 110-kDa catalytic subunit with specificity toward the D3 hydroxyl in the inositol ring of phosphatidylinositol (37, 54, 81, 98). Numerous studies provide evidence that PI3K, in association with various mitogenically active receptor and nonreceptor protein tyrosine kinases, mediates the transmission of growth-regulatory information within cells (reviewed in references 18, 21, and 82). These studies suggest that the activation of PI3K contributes a positive, but undefined, cell proliferation signal.

The activity of the 70/85-kDa S6 protein kinases (αI and αII isoforms, referred to collectively as pp70^{S6k}) is also stimulated by IL-2 in IL-2-responsive cells (16, 63, 106) as well as in other cell types by many growth factors and oncogenes (reviewed in reference 36). However, the cytosolic mediators involved in its signal cascade have been previously unknown. pp70^{S6k} was identified on the basis of its ability to phosphorylate the 40S ribosomal protein S6 in vitro. A number of other kinases, including the growth-regulated 90-kDa ribosomal S6 kinases (RSKs), can also phosphorylate S6 in vitro, but evidence indicates that in vivo, this function is performed by pp70^{S6k} (28). Recent data suggest that the pathways leading to pp70^{S6k} activation and the subsequent phosphorylation of S6 are important for growth factor-modulated G_1 transition in fibroblasts, hepatoma cells, and T lymphocytes (16, 28, 63, 65, 85, 86, 106). Activation of pp70^{S6k} is associated with phosphorylation of several clustered Ser/Thr-Pro residues in its putative pseudoinhibitory domain (5, 40). Identification of the kinase and/or phosphatase activities responsible for regulating the phosphorylation status of these residues has been unsuccessful (77). Additionally, several studies suggest that the signalling system regulating pp70^{S6k} is distinct from another well-characterized signal transduction system which includes c-Ras, c-Raf, MEK, mitogen-activated kinase kinase (MAPK), and RSK (reviewed in references 9, 30, 31, and 68).

We have recently found that PI3K participates in platelet-

* Corresponding author. Mailing address: Department of Cell Biology, Harvard Medical School, 25 Shattuck St., Boston, MA 02115. Phone: (617) 432-4848. Fax: (617) 432-1144.

† Present address: Program in Biologic Sciences, University of California, San Francisco, San Francisco, CA 94143.

derived growth factor (PDGF)- and insulin-dependent activation of pp70^{S6k} (23, 27). Activation of PI3K by PDGF or insulin is mediated by receptor-tyrosine kinase activation and phosphorylation of the receptor (for PDGF) or IRS-1 (for insulin). These tyrosine-phosphorylated proteins act as docking structures for the recruitment and activation of PI3K through its regulatory subunit SH2 domains (83). IL-2 has been shown to activate PI3K (4, 71, 87), although the extent (fold) of activation is small in comparison with what is observed with PDGF or insulin in other cell systems (18, 45). The mechanism for PI3K activation by IL-2 is unclear, but the available evidence indicates that it is different from that described above. Unlike the PDGF and the insulin receptors, the IL-2R lacks any intrinsic protein tyrosine kinase activity. Still, IL-2-dependent stimulation of PI3K is accompanied by association of PI3K activity with the IL-2R β chain and tyrosine phosphorylation of the 85-kDa regulatory subunit of PI3K (71, 87). Both activation of PI3K and association with the IL-2R appear to be dependent on tyrosine kinase activity (71) as well as on the serine-rich domain of the IL-2R β chain (72), which has been shown to be required for IL-2-dependent proliferation (50, 51). The Src family nonreceptor tyrosine kinases p56^{lck} and p59^{fyn} have been proposed as potential mediators of the IL-2-dependent activation of PI3K (59, 102).

While the immunosuppressant rapamycin potently and specifically inactivates pp70^{S6k} in all cell types and in response to all activating agents tested so far (16, 28, 42, 63, 85, 106, 110), its inhibitory effects in the cell cycle (G_1-S transition) are particularly marked in IL-2-dependent cells. This finding suggests that pp70^{S6k} activity is particularly important for T-cell proliferation and of particular significance with regard to immunosuppression and immune system disorders. Interestingly, a *Drosophila melanogaster* S6 knockout results in the overgrowth of lymph glands, abnormal blood cell differentiation, and melanotic tumor formation, consistent with a role for the regulation of the pp70^{S6k} pathway in proper hematopoietic system growth and differentiation (100, 113). This finding, together with the role of PI3K as a pp70^{S6k} upstream regulator in other systems, prompted us to determine if IL-2-dependent signalling to the pp70^{S6k} phosphorylation cascade is mediated by PI3K in lymphoid cells.

Inhibition of IL-2-dependent G_1-S transition is also observed in response to elevation of intracellular cyclic AMP (cAMP) levels (26, 34). cAMP is a ubiquitous second messenger implicated in positive and negative modulation of cell growth. Early data suggested that increased intracellular cAMP levels inhibited T-cell activation (reviewed in reference 27). More recent work, however, indicates that this inhibition is mediated through blocking IL-2-dependent G_1-S transition (56, 66). It is well established that elevated cAMP directly results in the activation of cAMP-dependent protein kinase (PKA), a broad-specificity serine/threonine protein kinase. While the mechanism of PKA activation by cAMP is well characterized (reviewed in references 104 and 105), the mechanism by which PKA then mediates signal transduction is poorly understood. Because of the biological effects of raising cAMP in lymphoid cells, we were also interested in determining if PKA signalling might antagonize IL-2-dependent pp70^{S6k} activation.

Here we demonstrate that IL-2-dependent activation of PI3K regulates the serine/threonine phosphorylation cascade within which pp70^{S6k} functions, thus establishing PI3K as the first functional component described so far between the IL-2R and pp70^{S6k}. This signalling process is independent of conventional protein kinase C (cPKC) isoforms. Activation of PKA inhibits pp70^{S6k} activation by interfering at distinct points in this signalling system. Activated PKA antagonizes IL-2-dependent PI3K activation and inhibits downstream of cPKC, possibly after a point of convergence of the PI3K- and cPKC-regulated paths to pp70^{S6k}. Further, we report that rapamycin, which presumably interferes with an upstream modulator of pp70^{S6k}, acts downstream of PI3K and, like cAMP, at or after a point where the PI3K- and cPKC-regulated pathways converge. Alternatively, it acts on a pathway involved in the regulation of pp70 that is independent of PI3K and cPKC. These possibilities are of interest since a putative target of rapamycin (TOR) which has homology with PI3K has recently been identified (15, 52, 62). However, TOR lipid or protein kinase activity has not yet been described. Although we do not define the mechanism of TOR action, we have ruled out PKA activation. Surprisingly, activation of PKA has no effect on IL-2-dependent activation of MAPK or RSK.

MATERIALS AND METHODS

Materials. Tissue culture media, sera, and additives were purchased from GIBCO (Grand Island, N.Y.). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), phorbol myristate acetate (PMA), wortmannin, Kemptide, protein kinase inhibitor, Nonidet P-40, β-mercaptoethanol, pepstatin, leupeptin, penicillin-streptomycin solution, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, Mo.). PMA was from LC Laboratories (Woburn, Mass.). Enhanced chemiluminescence reagents were obtained from Amersham (Amersham, England). Recombinant IL-2 and rapamycin were kindly provided by M. K. Gately (Hoffman-La Roche, Nutley, N.J.) and S. N. Seghal (Wyeth-Ayerst), respectively. LY294002 was provided by Eli Lilly.

Cell culture. Two murine IL-3-dependent pro-B-cell lines ectopically expressing the high-affinity wild-type human IL-2R (Baf α/β) or a deletion mutant of the human IL-2R β chain lacking the serine-rich domain, amino acids 267 to 322 (Baf α/β_{SD-1}), were kindly provided by W. C. Greene (University of California, San Francisco). They were cultured in RPMI 1640–10% fetal bovine serum–10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.2)–2 mM glutamine–50 μM β-mercaptoethanol–20 U of penicillin per ml–20 μg of streptomycin per ml supplemented with 10% WEHI.3B-conditioned medium as described previously (72). The murine IL-2-dependent CTLL-20 cell line was cultured in Dulbecco modified Eagle medium supplemented with L-arginine HCl, L-asparagine–H_2O, folic acid, essential vitamins, nonessential amino acids, 10% (vol/vol) heat-inactivated fetal bovine serum, 10 mM HEPES (pH 7.2), 2 mM glutamine, 50 μM β-mercaptoethanol, 20 U of penicillin per ml, 20 μg of streptomycin per ml, and 10 U of recombinant human IL-2 (referred to as IL-2) per ml. Cells were starved of IL-3 or IL-2 for 14 to 18 h before treatment with drugs and/or stimulation with IL-2 (100 U/ml) or PMA (100 ng/ml).

Antibodies. Previously generated antibodies to the predicted amino acids 20 to 39 and 502 to 525 of pp70^{S6k} were used for immune complex kinase assays. Only the anti-C-terminal antibody was used for immunoblot analysis (28, 47). Antibodies against RSK have been previously described (25). Antiphosphotyrosine antibodies were kindly provided by B. Druker and T. M. Roberts.

Protein kinase assays. Cells were stimulated for 30 or 45 min to determine pp70^{S6k} activity or for 10 min to determine RSK and MAPK activities. Ribosomal 40S subunits used for pp70^{S6k} (78) or RSK assays were purified from rat liver as described previously (107). Cells were lysed in cold cell lysis buffer (11), and the lysates were cleared by centrifugation for 10 min at 13,000 × *g*. The immune complex kinase assays were performed as described previously (24). Phosphotransferase activities were quantified with a PhosphorImager apparatus or by liquid scintillation counting.

A PKA in vitro assay was modified from those described previously (88, 114). Lysate (5 μl) was added to 20 μl of assay cocktail (50 μM ATP, 10 μCi of [γ-^{32}P]ATP, 10 mM $MgCl_2$, 20 mM HEPES [pH 7.2], 0.1 mg of BSA per ml, 3 mM β-mercaptoethanol, 3 μg of Kemptide per assay) with or without 31.25 μM cAMP or 2.25 μM protein kinase inhibitor, and the reaction was allowed to continue for 10 min at 30°C before addition of perchloric acid (3 ml). Samples were then spotted on P81 phosphocellulose paper, washed four times with 75 mM phosphoric acid and once each with water and ethanol, then dried for 30 min at 50 to 60°C, and counted in a liquid scintillation counter. Results are shown for each condition as a percentage of the maximal PKA activity obtained when excess cAMP (31.25 μM) was added to in vitro assay minus the basal phosphate incorporation into Kemptide as determined by the addition of excess protein kinase inhibitor (2.25 μM) to the in vitro assay cocktail.

PI3K assay. PI3K assays were performed as described previously (115), with minor modifications. Cells stimulated with IL-2 (1,000 U/ml) for 7.5 min were lysed, and PI3K activities were immunoprecipitated with antiphosphotyrosine antibody 4G10. The phosphotransferase assays were performed for 15 min at 30°C and terminated by addition of 200 ml of 1 N HCl. Phosphorylated phosphoinositides were separated by thin-layer chromatography on a Silica Gel 60 plate (Whatman) that was impregnated with 1% potassium oxalate. $CHCl_3$–

methanol–4 M NH_4OH (9:7:2) or *n*-propanol–2 M acetic acid (1.8:1) solution was used as a developing solvent for thin-layer chromatography analysis. Phosphotransferase activity was quantified with a PhosphorImager apparatus.

Immunoblot analyses. Immunoblot analyses were performed as described previously (28). Aliquots of the cleared lysates containing 20 to 50 μg of cellular proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE; 7.5% polyacrylamide gel) and electrically transferred to a nitrocellulose membrane. $pp70^{S6k}$ was detected by the enhanced chemiluminescence method, using rabbit polyclonal anti-$pp70^{S6k}$ antiserum as the primary antibody (1:2,000 dilution).

Cell proliferation. CTLL-20 cells were plated on 60-mm-diameter dishes and grown for 34 h in complete medium plus IL-2 and then starved of IL-2 for 14 h prior to IL-2 (100 U/ml) stimulation with or without treatment with forskolin-IBMX or rapamycin. Cells were pulsed with [^{3}H]thymidine (1 μCi per plate) for 2 h and then prepared as described previously (28).

RESULTS

Differential IL-2-dependent stimulation of mitogen-activated Ser/Thr kinases; $pp70^{S6k}$ activation is independent of cPKCs in T cells. The IL-2R signalling cascade utilizes many of the same cytosolic components as other cytokine and growth factor receptors, including many members of the MAPK signalling cascade such as c-Ras, c-Raf, MEK, and RSK. $pp70^{S6k}$, which can be regulated by Ras-independent signalling pathways (8), is also activated upon IL-2 stimulation. However, the signalling molecules that lie between the IL-2R and downstream signal transducers such as $pp70^{S6k}$ remain undefined. In other signalling systems, phospholipase Cγ, which regulates cPKCs, may function between growth factor receptors and signalling kinases such as MAPK and $pp70^{S6k}$. However, it has been shown that IL-2R-mediated signalling and proliferation is independent of phospholipase Cγ and cPKCs (61, 73, 74, 91, 109, 111; reviewed in reference 19). IL-2-dependent T-cell lines such as CTLL-20 cells contain functional cPKCs which can be activated by phorbol esters such as PMA. Interestingly, both PMA and IL-2 stimulated $pp70^{S6k}$ to approximately the same extent in CTLL-20 cells, whereas there was a dramatic difference in the activation of MAPKs and RSK by PMA and IL-2 (Fig. 1). IL-2 is a weak agonist for MAPK and RSK in comparison with PMA. Analogous results were observed in several other murine and human IL-2-responsive lymphoid cell lines, including Baf α/β, CTLL-2, and Kit225 (not shown). Importantly, inhibition of cPKC by downregulation through PMA pretreatment had no effect on the extent of $pp70^{S6k}$ activation by IL-2 (Fig. 2). We conclude that IL-2 activates $pp70^{S6k}$ in a cPKC-independent manner. Since IL-2-dependent activation of $pp70^{S6k}$ is not dependent on cPKC or the Ras-regulated protein kinase pathway, we examined whether PI3K might mediate the IL-2-dependent signal transduction to $pp70^{S6k}$.

An IL-2R defective in PI3K activation does not regulate $pp70^{S6k}$. To investigate the possibility that PI3K is an upstream regulator of $pp70^{S6k}$, we first used two IL-3-dependent murine pro-B-cell lines expressing wild-type IL-2R (Baf α/β; expressing normal α, β, and γ subunits) and a mutant IL-2R (Baf α/β_{SD-1}; wild-type α and γ subunits with a mutant β subunit). The mutant receptor β subunit is missing its serine-rich domain, which renders it unable to activate PI3K in response to agonist (72). Although this type of mutant receptor can still bind to the cytoplasmic tyrosine kinase Lck (50), it is not able to support IL-2-dependent cell proliferation (51, 72). Addition of IL-2 to cells expressing the wild-type IL-2R resulted in a severalfold activation of $pp70^{S6k}$ (Fig. 3), as previously observed in this (Fig. 1 and 2) and other IL-2-responsive cell culture systems (16, 63, 106). As expected, activation of $pp70^{S6k}$ was accompanied by its phosphorylation at several sites, as determined by its reduced mobility on SDS-gels (not shown). In contrast, IL-2 did not activate $pp70^{S6k}$ in cells expressing the mutant IL-2R (Fig. 3). Phorbol ester-mediated activation of cPKC (Fig. 3) or stimulation of the cells with IL-3 (not shown) resulted in the stimulation of $pp70^{S6k}$ in both cell lines. These results provide a correlation between the activity of PI3K and that of $pp70^{S6k}$ and suggest that the enzymes may be functionally coupled.

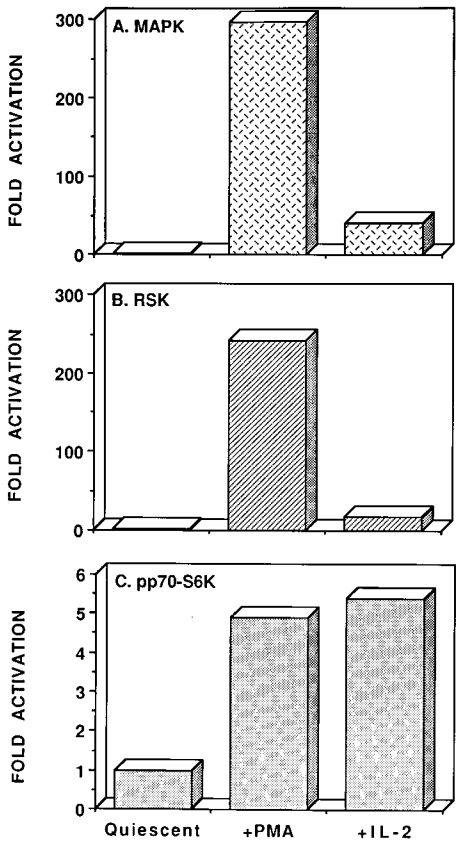

FIG. 1. Differential stimulation of MAPK, RSK, and $pp70^{S6k}$ by IL-2 and PMA in T cells. Quiescent CTLL-20 cells were either left untreated or stimulated with PMA or IL-2 for 10 min (for analysis of MAPK and RSK activities) or 45 min (for $pp70^{S6k}$) before being lysed. Immune complex kinase assays were carried, out and enzymatic activities were measured with a PhosphorImager after SDS-PAGE. The results are presented for each enzyme as fold stimulation relative to the activity levels in quiescent cells.

PI3K inhibitors block IL-2-dependent activation of $pp70^{S6k}$. It is possible that signal transduction molecules other than PI3K could be affected by the deletion of the serine-rich region in the IL-2R β chain. Therefore, we used an independent approach to address the putative relationship between PI3K and $pp70^{S6k}$. The steroid-like compound wortmannin has recently been shown to be a highly specific and potent inhibitor of PI3K, inhibiting PI3K in vitro and in vivo and preventing production of phosphatidylinositol-3,4,5-P_3 in vivo with a 50% inhibitory concentration (IC_{50}) of 1 to 10 nM (2, 58, 117). Although wortmannin did not inhibit $pp70^{S6k}$ activity in vitro (not shown), it potently blocked IL-2-dependent activation of $pp70^{S6k}$ in CTLL-20 cells with an IC_{50} of 1 to 10 nM (Fig. 4A). As previously shown with rapamycin, wortmannin blocked phosphorylation of $pp70^{S6k}$ (Fig. 5). A structurally unrelated compound, LY294002 (a bioflavonoid related to quercetin), has also been shown to be a highly specific inhibitor of PI3K, with an IC_{50} of 1 to 10 μM (112). Like wortmannin, LY294002 does not inhibit any protein kinase tested to date, nor does it inhibit PI4K at concentrations inhibiting PI3K (112, 117).

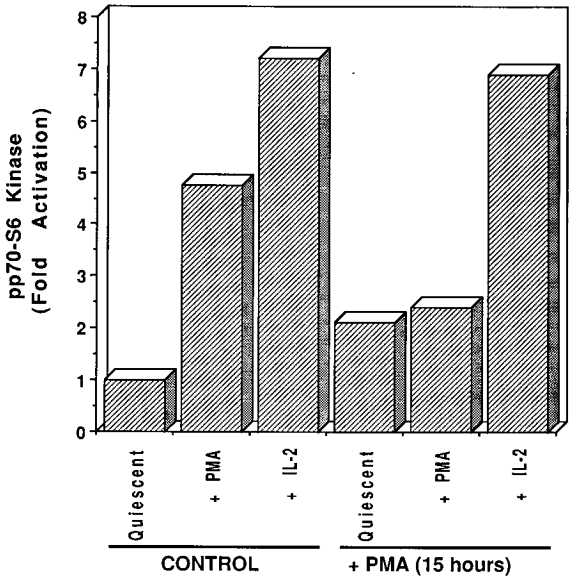

FIG. 2. IL-2-dependent pp70^{S6k} activation is independent of cPKCs. CTLL-20 cells were incubated overnight in medium minus IL-2 with or without PMA (100 ng/ml). Quiescent (control) and quiescent, down-regulated (+ PMA) cells were stimulated with IL-2 or PMA for 45 min or left untreated. pp70^{S6k} activity was measured by immune complex assays of the cleared lysates and quantified with a PhosphorImager after SDS-PAGE. The results are presented as fold stimulation relative to the activity levels in the quiescent, not PMA-down-regulated controls.

LY294002 blocked IL-2-dependent activation of pp70^{S6k} with the same IC_{50} as shown for inhibition of PI3K (Fig. 4B). Because of the structural differences between these drugs, it is most likely that inhibition of pp70^{S6k} activation is due to their common property of inactivating PI3K rather than to a shared nonspecific effect.

Phorbol ester-mediated activation of pp70^{S6k} is independent of PI3K. Although IL-2-dependent activation of pp70^{S6k} is independent of cPKC (Fig. 2), we have shown that pp70^{S6k} can

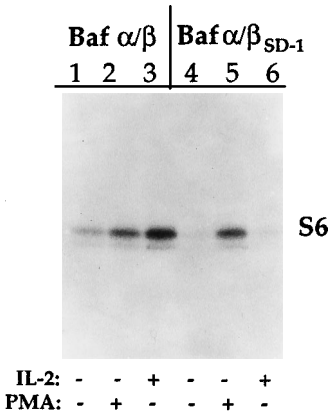

FIG. 3. IL-2-dependent activation of pp70^{S6k} requires an IL-2R β-chain region necessary for PI3K activation. Baf cells expressing either wild-type IL-2R β chain (lanes 1 to 3) or a deletion mutant of IL-2R β chain at the serine-rich domain (lanes 4 to 6) were starved of IL-3 and stimulated with IL-2 (lanes 3 and 6) or PMA (lanes 2 and 5) for 45 min. Cell lysates were prepared from the same number of cells (2×10^6), and the S6 phosphotransferase activity of pp70^{S6k} was assessed by immune complex kinase assays. Immunoblot analysis showed that the lysates from both cell lines contained equivalent amounts of pp70^{S6k} (not shown).

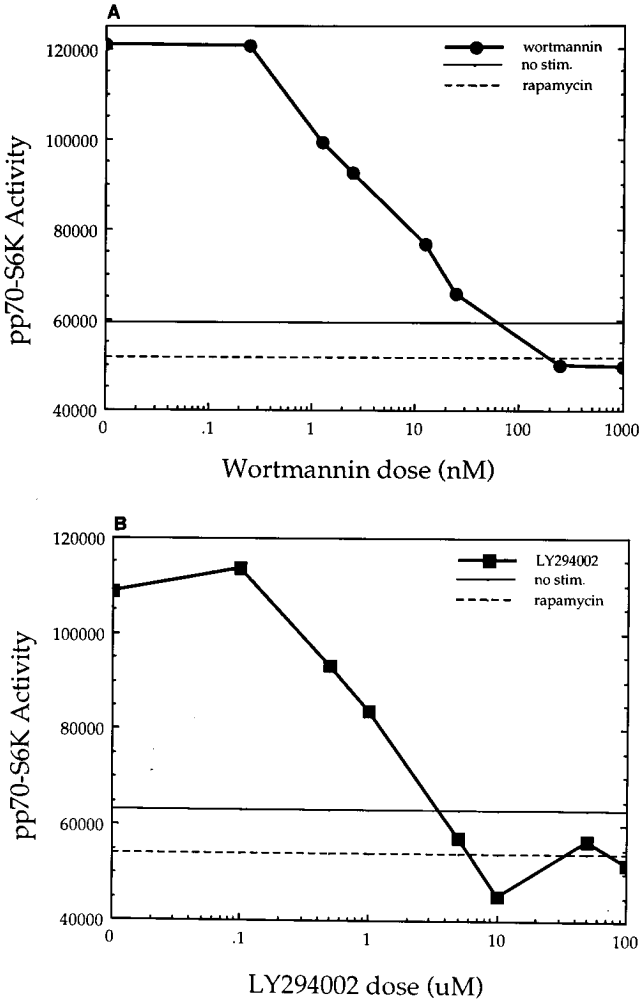

FIG. 4. Two PI3K inhibitors, wortmannin and LY294002, inhibit IL-2-dependent activation of pp70^{S6k} in vivo. CTLL-20 cells starved of IL-2 were treated with the vehicle dimethyl sulfoxide (zero drug concentration) or various concentrations of either wortmannin for 5 min (A) or LY294002 for 10 min (B) and then stimulated with IL-2 for 45 min before lysis. The S6 phosphotransferase activity of pp70^{S6k} was measured by immune complex assays. ^{32}P incorporation into the S6 substrate was quantified following SDS-PAGE and presented in PhosphorImager arbitrary units as a dose-response curve. Stimulation of pp70^{S6k} by IL-2 was also inhibited by a 5-min pretreatment with rapamycin (20 ng/ml), as indicated by the dashed line. Activity levels in quiescent cells are indicated.

be activated by phorbol esters in lymphoid cells (Fig. 1C). We then tested whether cPKC-dependent activation of pp70^{S6k} in lymphoid cells required PI3K. To do so, we incubated Baf α/β and CTLL-20 cells with or without wortmannin before stimulating them with IL-2 or PMA. Although wortmannin completely inhibited IL-2-dependent phosphorylation and activation of pp70^{S6k}, it was without effect on PMA-dependent phosphorylation and activation of pp70^{S6k} in these cells (Fig. 5). In contrast, rapamycin potently blocked activation of pp70^{S6k} by both agonists (Fig. 5B). These results are consistent with wortmannin and rapamycin blocking distinct points in the signalling pathway regulating pp70^{S6k} activity.

Phosphorylation/dephosphorylation of pp70^{S6k} is under dynamic regulation. Previous experiments with rapamycin have shown that its addition to stimulated cultured cells in which pp70^{S6k} is already maximally activated results in its rapid dephosphorylation and inactivation (28, 41, 63). Although we

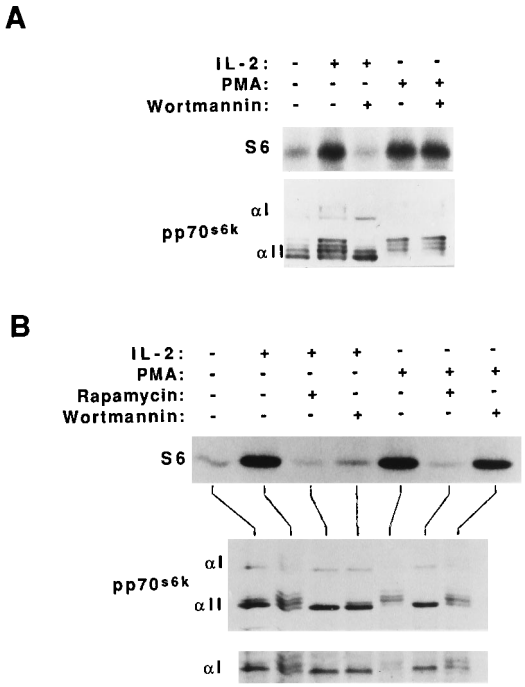

FIG. 5. Wortmannin inhibits IL-2-stimulated but not PMA-stimulated pp70^{S6k} activity. (A) Baf α/β cells expressing the wild-type IL-2R were starved and then treated for 5 min with either wortmannin (50 nM) or the vehicle dimethyl sulfoxide. (B) CTLL-20 cells were starved and treated for 5 min with wortmannin (100 nM) or rapamycin (20 ng/ml). In both assays, cells were then stimulated with either IL-2 or PMA for 45 min before lysis. Gels labeled S6 are autoradiographs of the S6 substrate after immune complex assay of pp70^{S6k} phosphotransferase activities. Gels labeled pp70^{S6k} (αI and αII) show the pp70/85^{S6k} immunoblot analysis performed on the lysates. In panel B, the lower gel is a longer exposure of the upper gel, which clearly demonstrates that the extent of αI phosphorylation parallels that of αII phosphorylation is these experiments.

have provided data that this is not the result of activating PP1C or PP2A (28), it is possible that another, highly specific phosphatase is activated. Alternatively, if there is dynamic regulation of pp70^{S6k} by both active kinases and phosphatases, then inactivation of upstream kinases may also result in the rapid dephosphorylation of pp70^{S6k} that is observed. From the experiments presented in Fig. 5, it appears that PI3K is further upstream in the pp70^{S6k} signalling pathway than is the rapamycin target. We then examined how pp70^{S6k} activity would be affected after inhibiting PI3K in IL-2-stimulated cells. As shown in Fig. 6, addition of wortmannin resulted in the rapid inactivation of pp70^{S6k}. Inactivation back to basal-activity levels was measured within 5 min with rapamycin and 15 min with wortmannin. The differences in the rates of inactivation support the evidence provided above that these reagents target different points along this signalling pathway. These results also support the notion that phosphorylation of both pp70^{S6k} and pp85^{S6k} is carefully modulated by regulated upstream kinases and highly active phosphatases. Indeed, all enzymes involved in the coupling of PI3K to pp70^{S6k} would appear to be under similar dynamic regulation. Since pp85^{S6k} is nuclear and pp70^{S6k} is cytoplasmic and nuclear (86), the putative pp70/85^{S6k} phosphatases must be similarly localized. Additionally, our data indicate that PI3K activity is needed to maintain pp70^{S6k} in an activated state throughout this time course.

Increased cAMP antagonizes IL-2-mediated pp70^{S6k} regulation. Treating IL-2-dependent T cells with agents to raise intracellular cAMP level at the time of or prior to stimulation with IL-2 blocks IL-2-dependent phosphorylation and activation of pp70^{S6k} in a dose-dependent manner (Fig. 7A to D). Rapamycin also inhibits IL-2-dependent activation of pp70^{S6k} (16, 63, 106) (Fig. 7A, B, and E). Either raising the cAMP level or treating cells with rapamycin antagonizes IL-2-dependent T-cell proliferation, as measured by reduced [^{3}H]thymidine uptake, a marker of G_1-S phase transition (Fig. 7E) (28, 56). Our data for increased cAMP present yet another example that inhibition of IL-2-dependent G_1 pp70^{S6k} activation correlates with a block in the G_1-S transition in T cells.

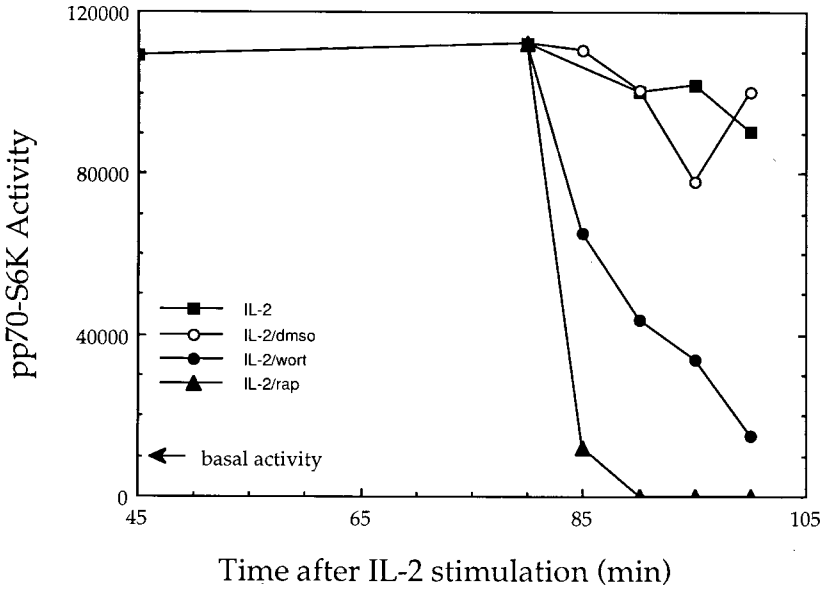

FIG. 6. Rates of inactivation of pp70^{S6k} by wortmannin and rapamycin. CTLL-20 cells in which pp70^{S6k} was maximally activated by IL-2 stimulation were treated for different periods of time, starting 80 min after IL-2 addition, with wortmannin (wort; 100 nM), rapamycin (rap; 20 ng/ml), or dimethyl sulfoxide (dmso; vehicle) before being lysed. The S6 phosphotransferase activity of pp70^{S6k} was measured by immune complex assays. ^{32}P incorporation into the S6 substrate was quantified following SDS-PAGE and presented in PhosphorImager arbitrary units.

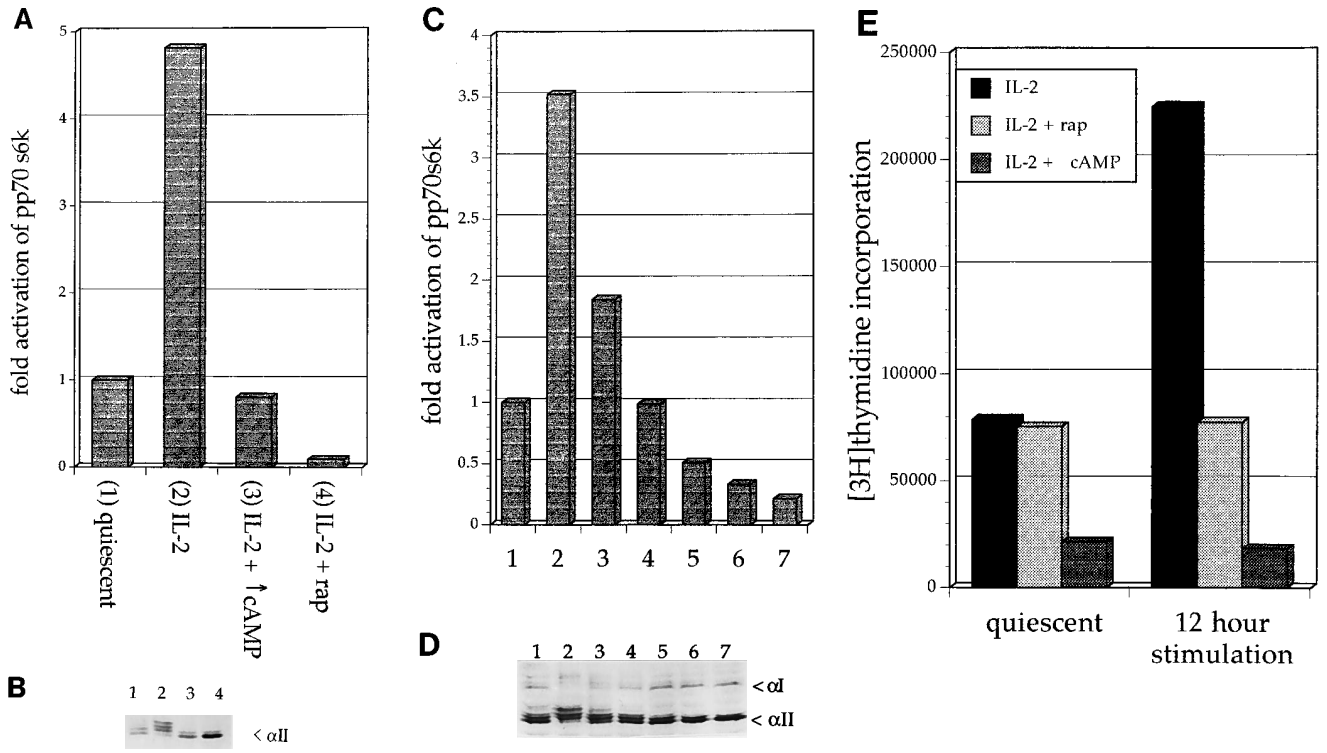

FIG. 7. Agents that increase intracellular cAMP inhibit IL-2-dependent activation of pp70^{S6k} and block the G_1-S transition in CTLL-20 cells. (A) Inhibition of IL-2-activated pp70^{S6k} in CTLL-20 cells by agents that increase intracellular cAMP. CTLL-20 cells were starved for 14 to 18 h in complete medium minus IL-2 and then treated for 45 min prior to lysis with IL-2 (100 U/ml), IL-2 (100 U/ml), forskolin (25 μM), and IBMX (500 μM) (IL-2 + ↑cAMP), or IL-2 (100 U/ml) plus rapamycin (rap; 20 ng/ml) as indicated. pp70^{S6k} activity was assayed with an in vitro immune complex kinase assay. Activity is expressed as fold activation of pp70^{S6k} in relation to quiescent levels. (B) Dephosphorylation of pp70^{S6k} by agents that increase intracellular cAMP. In an immunoblot analysis of the lysates from panel A, the more slowly migrating form of pp70^{S6k} correlates with the phosphorylated, activated enzyme. Only the αII form (pp70^{S6k}) is shown. Similar results were observed with the αI (p85^{S6k}) form. (C) Dose-dependent inactivation of pp70^{S6k} by forskolin and IBMX. CTLL-20 cells were starved of IL-2 for 17 h. Treatment: 1, none (quiescent); 2, IL-2 (100 U/ml); 3, IL-2 (100 U/ml), forskolin (6.25 μM), and IBMX (125 μM); 4, IL-2 (100 U/ml), forskolin (12.5 μM), and IBMX (250 μM); 5, IL-2 (100 U/ml), forskolin (18.75 μM), and IBMX (375 μM); 6, IL-2 (100 U/ml), forskolin (25 μM), and IBMX (500 μM); 7, IL-2 (100 U/ml), forskolin (50 μM), and IBMX (1 mM). (D) Dose-dependent dephosphorylation and mobility shift of pp70^{S6k} by forskolin and IBMX. An anti-pp70^{S6k} immunoblot of lysates from the forskolin-IBMX dose response in panel C is shown. Both αII and αI forms are indicated. (E) Elevation of intracellular cAMP blocks IL-2-dependent G_1-S transition in CTLL-20 cells. Cyclic AMP blocks incorporation of [^{3}H]thymidine in IL-2-stimulated CTLL-20 cells. Cells were starved and then stimulated with IL-2 with or without forskolin-IBMX or rapamycin (rap) as described above. Ten hours after stimulation, cells were treated with 1 μCi of [^{3}H]thymidine until 12 h poststimulation, when they were lysed. Following trichloroacetic acid precipitation and washes, lysates were counted in a scintillation counter.

Cotreatment with forskolin, a diterpine that activates adenylate cyclase, and IBMX, an inhibitor of the cAMP phosphodiesterase, of cells which had been stimulated for 45 min with IL-2 (resulting in maximal pp70^{S6k} activity) led to a rapid inactivation and dephosphorylation of pp70^{S6k} (Fig. 8). Within 5 to 10 min of raising the intracellular cAMP level, pp70^{S6k} activity was back to levels in quiescent cells. This result was reminiscent of the rate of inactivation observed with rapamycin and wortmannin (28).

While rapamycin is an exogenous agent, raising the intracellular cAMP levels is a potential physiologic route for regulating IL-2-dependent T-cell responses. As the mechanism has not been established for either rapamycin- or cAMP-induced inhibition, we examined whether rapamycin activated PKA or whether increased cAMP effects were mediated by the rapamycin receptor, FKBP.

Increased cAMP, but not rapamycin, activates PKA. It has been shown that IL-2 stimulation does not activate PKA in T cells (Fig. 9 and reference 91). Incubation of cells with forskolin and IBMX leads to the activation of PKA (Fig. 9), consistent with stimulation seen in a prior study of peripheral blood lymphocytes with cell-permeable analogs of cAMP (66). Importantly, rapamycin treatment did not activate PKA (Fig. 9). Presumably PKA's downstream effects are involved in blocking the phosphorylation and activation of pp70^{S6k}; however, these events are poorly characterized. Whether rapamycin modulates events downstream of PKA is not known. We next examined whether cAMP affected the initial intracellular target of rapamycin, FKBP.

Inhibition of pp70^{S6k} by cAMP is not reversed by FK506. Rapamycin requires binding to its ubiquitous intracellular receptor FKBP, a *cis-trans* peptidyl-prolyl isomerase, to cause immunosuppression (49, 97; reviewed in references 92 and 94). At present, no endogenous ligand for the rapamycin-binding site on FKBP has been identified. The immunosuppressant FK506, which inhibits T-cell G_0-G_1 transition, also requires binding to FKBP at the same site as rapamycin (reviewed in references 92 to 94). FK506 alone has no effect on pp70^{S6k} activity. The FK506-FKBP complex binds to and inhibits the calcium/calmodulin-dependent phosphatase calcineurin, thus preventing dephosphorylation and nuclear translocation of the NF-AT cytoplasmic subunit, a necessary step in IL-2 gene transcription (43, 67). However, when added in 500- to 2,000-fold molar excess, FK506 functions as a competitive antagonist for rapamycin's physiologic actions, including reversing rapamycin's inhibition of pp70^{S6k} (7, 28, 35). We speculated that if increasing intracellular cAMP and activating PKA generated or activated an endogenous rapamycin homolog, then excess

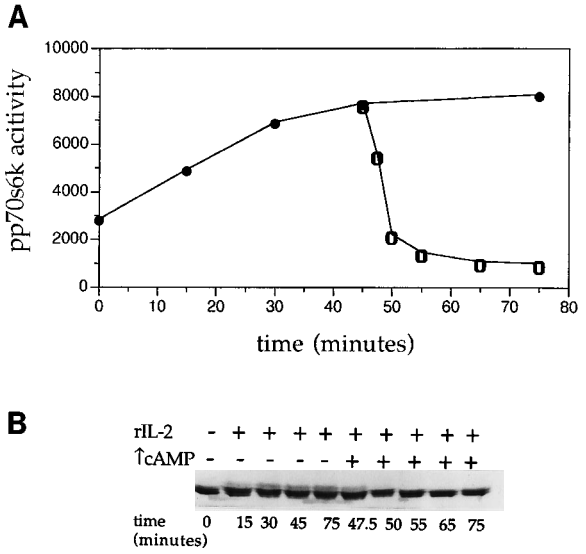

FIG. 8. Effect of cAMP-raising agents on fully activated pp70^{S6k}. (A) Increasing cAMP rapidly inactivates pp70^{S6k}. Cells were starved of IL-2 for 16 h. They were treated with IL-2 (100 U/ml) for 0, 15, 30, 45, and 75 min prior to lysis (●) or with IL-2 for 45 min, after which forskolin (25 μM)–IBMX (500 μM) was added for 2.5, 5, 10, 20, or 30 additional min prior to lysis (○). Fully activated pp70^{S6k} was inactivated to quiescent levels within 5 min of drug treatment. (B) Increasing cAMP rapidly dephosphorylates pp70^{S6k}. Aliquots of the lysates described for panel A were analyzed by immunoblotting with pp70^{S6k} antiserum. A rapid reversal of the phosphorylation-dependent change in mobility of pp70^{S6k} on an SDS–7.5% gel was detected. Only the αII form is shown.

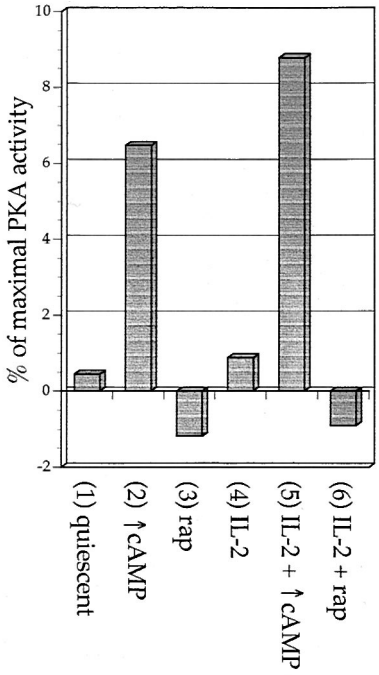

FIG. 9. Raising the intracellular cAMP level activates PKA. CTLL-20 cells were starved for 16 h in complete medium minus IL-2 and then not treated (quiescent) or treated for 45 min prior to lysis with the following: 2, forskolin (25 μM) plus IBMX (500 μM); 3, rapamycin (20 ng/ml); 4, IL-2 (100 U/ml); 5, IL-2 (100 U/ml), forskolin (25 mM), and IBMX (500 μM); or 6, IL-2 (100 U/ml) plus rapamycin (20 ng/ml). Results were calculated as percentage of maximal PKA activity when excess cAMP was added to the in vitro assay minus background phosphate incorporation into Kemptide as determined by adding excess PKA to the in vitro assay.

FK506 might reverse the cAMP-induced inhibition of pp70^{S6k} by competitive binding to FKBP. This is not the case: FK506 has no effect on the ability of increased intracellular cAMP to quell pp70^{S6k} activity, whereas it fully reverses rapamycin's effect (Fig. 10).

One explanation for the inability of excess FK506 to reverse cAMP-induced pp70^{S6k} inhibition is that there may be no endogenous ligand for the FKBP-binding site. If, however, we assume the existence of an endogenous ligand which binds to FKBP and acts as a rapamycin homolog, then explanations for the above result include the following: (i) the endogenous homolog is not affected by heightened cAMP; (ii) it binds to a separate site on FKBP; or (iii) it binds to the same site as rapamycin or FK506 but cannot be competitively antagonized by FK506. At this time, we are not able to test these hypotheses. Having established that no apparent cross-talk between cAMP and rapamycin occurs at the level of their respective primary targets, we next compared the effects of each on known mechanisms of pp70^{S6k} regulation.

Increased cAMP inhibits cPKC-dependent and -independent activation of pp70^{S6k}. There are at least two signal transduction networks capable of activating pp70^{S6k}. One pathway is dependent on cPKC isoforms and sensitive to down-regulation by chronic treatment of cells with phorbol ester, while the other is independent of cPKCs and cannot be down-regulated by phorbol ester treatment (11, 25, 84, 101; PKC reviewed in reference 80). Observations from other groups suggest that the activation of PKA antagonizes PKC-dependent signalling pathways (79). On the basis of this information, we asked whether increasing intracellular cAMP would also inhibit cPKC-dependent activation of pp70^{S6k}. Addition of phorbol ester to cells resulted in activation of pp70^{S6k}, and this was antagonized by forskolin-IBMX treatment (Fig. 11A). Note that increased cAMP did not affect the cPKC-dependent activation of the 90-kDa RSK (1, 10, 24, 25), showing that the PKA inhibitory effects are not global (Fig. 11B and C). Previous studies have shown that cPKC down-regulation does not affect the ability of activated T cells to respond to IL-2 (61, 73, 74, 91, 111). Likewise, IL-2-dependent activation of pp70^{S6k} is insensitive to phorbol ester-mediated cPKC down-regulation (not shown). We conclude from our studies that agents which increase cAMP, thus activating PKA, antagonize both cPKC-dependent and -independent activation of pp70^{S6k}.

Activation of PKA inhibits IL-2-dependent activation of PI3K and pp70^{S6k} but not MAP kinase or RSK. Since we have provided evidence that IL-2-dependent activation of pp70^{S6k} is mediated by PI3K and since both the yeast and mammalian TORs resemble a lipid kinase similar to PI3K (15, 62), we examined whether cAMP or rapamycin blocked IL-2-dependent activation of PI3K. Under conditions in which IL-2 induced a severalfold activation of PI3K, activation of PKA resulted in inhibition of both PI3K (Fig. 12) and pp70^{S6k} (Fig. 11A and C). Under these conditions, rapamycin did not have a significant effect on PI3K (Fig. 12). Additionally, rapamycin in complex with FKBP-12 did not abolish PI3K activity measured in vitro (not shown). To determine if the IL-2R was no longer responsive to IL-2 in forskolin-IBMX-treated cells, we examined the IL-2-dependent activation of MAPK and RSK. IL-2 is a weak agonist of these signaling kinases, activating in the range of 2- to 10-fold (Fig. 11B and C and reference 38). For comparison, PMA activates these enzymes 30- to 100-fold in CTLL-20 cells, whereas IL-2 and PMA activate pp70^{S6k} approximately equally (Fig. 11). Activation of PKA had no effect on IL-2-mediated activation of MAPKs or RSK (Fig. 11B and

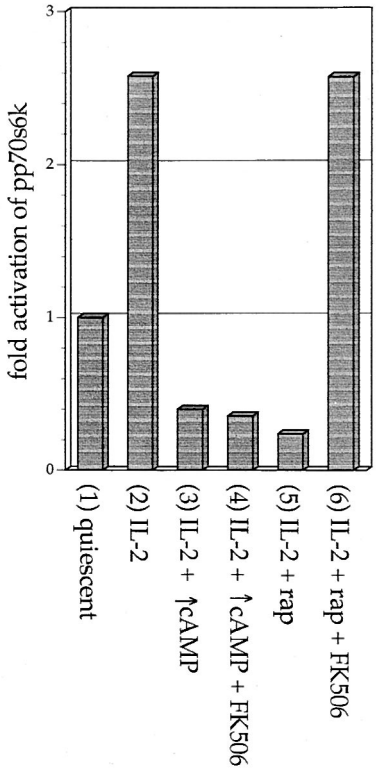

FIG. 10. Excess FK506 does not reverse cAMP-mediated inhibition of pp70^{S6k}. Cells were starved of IL-2 for 16 h then not treated (quiescent) or treated as follows prior to lysis: 2, IL-2 (100 U/ml); 3, IL-2 (100 U/ml), forskolin (25 μM), and IBMX (500 μM); 4, IL-2 (100 U/ml), forskolin (25 μM), IBMX (500 μM), and FK506 (8 μg/ml); 5, IL-2 (100 U/ml) plus rapamycin (rap; 4 ng/ml); and 6, IL-2 (100 U/ml), rapamycin (4 ng/ml), and FK506 (8 μg/ml). The 2,000-fold molar excess of FK506 reversed rapamycin's inhibition of IL-2-stimulated pp70^{S6k} activity, whereas it had no effect on cAMP-induced inhibition.

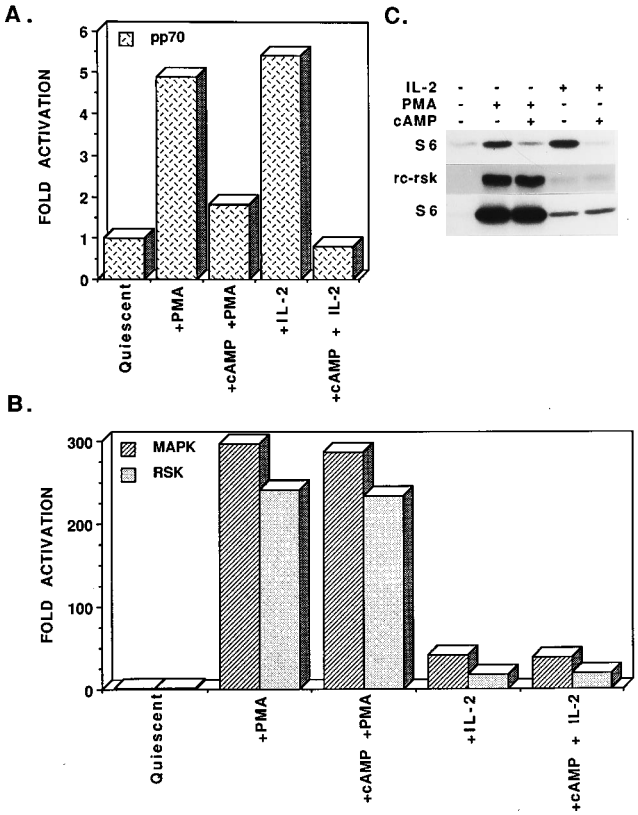

FIG. 11. Increased cAMP inhibits PKC- and IL-2-dependent activation of pp70^{S6k} but has no effect on RSK or MAPK. CTLL-20 cells which had been starved of IL-2 for 16 h were (i) left untreated (quiescent) or (ii) pretreated or not with forskolin (25 μM)–IBMX (500 μM) for 10 min and stimulated with PMA (100 ng/ml) or IL-2 (100 U/ml) for either 45 min (pp70^{S6k} immune complex assay) or 7.5 min (RSK and MAPK immune complex assays) prior to lysis. Activities of pp70^{S6k} (A) and of MAPK and RSK (B) are expressed as fold activation relative to quiescent cells. (C) Autoradiographs corresponding to the immune complex assays quantified in panels A and B, showing the phosphorylation of S6 substrate by pp70^{S6k} (top) and RSK (bottom) and of recombinant RSk protein (rc-rsk) by MAPK (middle).

C). Thus, the IL-2R is still capable of activating specific signal transduction pathways following forskolin treatment.

DISCUSSION

We provide evidence that the IL-2R-activated PI3K is coupled to the serine/threonine protein phosphorylation cascade involving the cytoplasmic and nuclear 70/85-kDa S6 kinases and the 40S ribosomal protein S6. In addition, we show that a complex negative cross-talk exists between cAMP-regulated signal transduction and this IL-2-stimulated path.

A complex signalling network couples PI3K to pp70^{S6k}. Using genetic and biochemical approaches, we show that PI3K is an important modulator of IL-2-dependent activation of pp70^{S6k}. Similar experiments have suggested that the PDGF and insulin receptors can also regulate pp70^{S6k} activity via PI3K (23, 27). However, the mechanism for activation of PI3K by IL-2 must be different from those used by these receptor tyrosine kinases, since the IL-2R has no intrinsic tyrosine kinase activity. Consistent with this notion is the observation that the activation of PKA antagonizes IL-2-dependent activation of PI3K and pp70^{S6k} but has little effect on PDGF-stimulated activation of both of these enzymes in HepG2 cells ectopically expressing the PDGF receptor (45). Activation of PKA has also been shown by others not to affect PI3K activation by insulin, epidermal growth factor, or PDGF (14). Furthermore, in light of recent evidence that PKA antagonizes MAPK activation and cell growth in several cell culture systems (14, 29, 46, 96, 116), it is of interest that in the lymphoid cells used, the activation of MAPK or RSK by phorbol esters as well as the small activation induced by IL-2 are both unaffected by PKA activation. The different effects of PKA activation on the MAPK pathway may be due to the differential use of certain signalling molecules like cAMP in different cell types, a notion

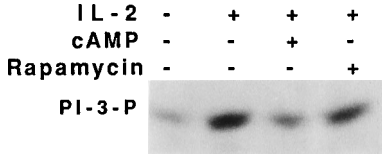

FIG. 12. Activation of PKA does, but rapamycin does not, inhibit IL-2-stimulated PI3K activity. PI3K activity from unstimulated and IL-2-stimulated CTLL-20 cells was immunoprecipitated from cell extracts with antiphosphotyrosine antibodies and measured as described in Materials and Methods. CTLL-20 cells were deprived of IL-2 for 12 h prior to treatment with cAMP-raising agents (forskolin [25 μM]–IBMX [500 μM]) or rapamycin (20 ng/ml) and/or stimulation with IL-2 (100 U/ml) for 7.5 min. Shown is a representative experiment. The means ± standard errors of the means ($n = 4$) of the fold PI3K activation relative to untreated controls were 2.65 ± 0.29 (+IL-2), 1.35 ± 0.10 (+cAMP +IL-2, a significant decrease at $P < 0.05$), and 2.08 ± 0.19 (+rapamycin +IL-2, not significant).

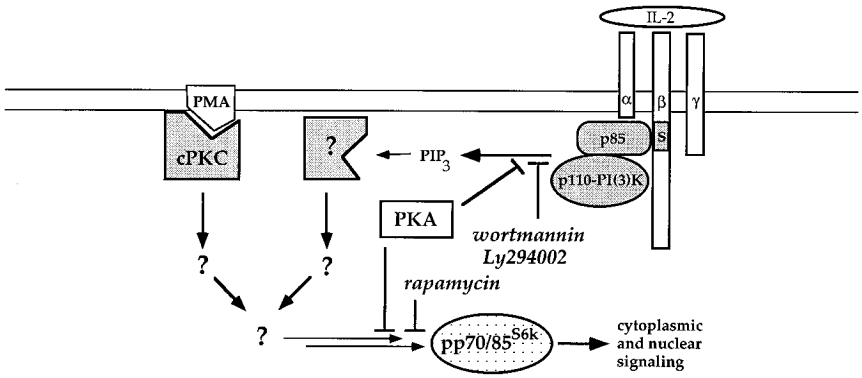

FIG. 13. Schematic representation of PI3K-dependent, cPKC-independent activation of pp70-S6 protein kinases. IL-2 binding to its receptor triggers the activation of PI3K and pp70/85^{S6k} in lymphoid cells. The serine-rich region of the IL-2R β chain (S) is necessary for these activations to take place. The results presented in this study indicate that PI3K is an upstream regulator of pp70/85^{S6k} and that the two proteins are linked through a cPKC-independent pathway. The question mark downstream of PIP_3 represents a possible mediator of signalling from PI3K to its downstream targets such as pp70^{S6k}. Such a mediator might be PKCζ (78). Also as a result of the experiments described, it is established that the target of rapamycin is not PI3K. PIP_3, phosphatidylinositol-3,4,5-P_3.

supported by the recent description of MAPK activation by cAMP in PC12 cells (44).

There are now several lines of experimental evidence linking inhibition of pp70^{S6k} in early G_1 with the failure of T cells to progress normally through G_1 and into S phase. In addition to published work with rapamycin (16, 28, 63), we show here that elevated cAMP-mediated inhibition of G_1 progression in IL-2-dependent T cells (56, 66) correlates with inhibition of pp70^{S6k}. Mutant T-cell lines in which cell proliferation is no longer sensitive to rapamycin have also been isolated. In these cells, pp70^{S6k} is no longer inhibited by rapamycin (34). Further, incubation of T cells with dexamethasone inhibits IL-2-dependent T-cell proliferation and also blocks pp70^{S6k} activation via a transcriptional mechanism (76). Consistent with the data obtained for lymphoid cells, addition of rapamycin to 3T3 fibroblasts prolongs G_1 transit (28), and microinjection of anti-pp70^{S6k} antibodies into rat fibroblasts also interferes with G_1 progression (65, 86). In spite of its potential relevance with regard to IL-2-dependent cell proliferation and T-cell immunosuppression, how pp70^{S6k} activity is regulated by IL-2 is not well understood.

Our data implicate PI3K and pp70^{S6k} as participating in the regulation of lymphoid IL-2-dependent G_1 cell cycle progression in a cPKC-independent manner. This finding is consistent with reports that IL-2 does not activate or at least does not require phospholipase Cγ and its downstream signalling events, including mobilization of intracellular calcium and activation of diacylglycerol-dependent cPKCs, to promote cell proliferation (19, 61, 65, 73, 74, 86, 91, 109, 111). Our results should now make it possible to design experiments aimed at identifying the intermediates in this signalling pathway and to determine how they interact as occurred after the demonstration that c-Ras is upstream of the MAPK phosphorylation cascade (9, 31, 68).

Wortmannin and rapamycin antagonize signal transduction to pp70^{S6k} at distinct points. In addition to the IL-2-dependent, PI3K-mediated activation of pp70^{S6k}, we present evidence derived from the experiments with wortmannin that in two lymphoid cell types, cPKC-mediated activation of pp70^{S6k} is independent of PI3K (Fig. 13). Rapamycin, however, blocks pp70^{S6k} activation by either phorbol esters or IL-2 and does not interfere with PI3K activity. These results suggest that the two pathways leading to pp70^{S6k} activation converge downstream of cPKC and PI3K but upstream of the target for the rapamycin-and-FKBP complex (Fig. 13).

In addition to cPKC, it is possible that Vav, a Ras guanine nucleotide exchange factor, participates in phorbol ester signalling to pp70 in hematopoietic cells (48). In such a case, wortmannin would not, but rapamycin would, antagonize this pathway, and Vav would also have to be sensitive to phorbol ester-mediated down-regulation.

cAMP-dependent protein kinase also interferes with IL-2 signalling to pp70^{S6k} at multiple points without affecting MAPK or RSK activation. Increasing intracellular cAMP levels has been described to strongly inhibit IL-2-mediated T-cell proliferation (56), and our data are consistent with the possibility that PKA activation antagonizes PI3K-mediated signalling in IL-2-responsive cells at at least two points. This multilevel cross-talk of PKA with a growth factor-activated pathway is reminiscent of the inhibitory effects of PKA activation on *Xenopus* oocyte maturation, which also occurs at multiple points (69).

Like rapamycin, PKA inhibits both the PI3K-dependent pp70^{S6k} activation in response to IL-2 and the cPKC-dependent pp70^{S6k} activation in response to phorbol ester. Unlike rapamycin, PKA is unlikely to act through the ubiquitous rapamycin receptors, the *cis-trans* peptidyl-prolyl isomerases referred to as FKBPs, nor is the mechanism of rapamycin inhibition through PKA activation. Inhibition is also not via direct inhibition of cPKC, since phorbol ester is still capable of activating the MAPK pathway in forskolin-treated cells. Thus, PKA activation appears to target a downstream effector common to both PI3K- and cPKC-mediated activation of pp70^{S6k} (this is illustrated in Fig. 13).

We also provide evidence that PKA-mediated inhibition of IL-2-dependent activation of pp70^{S6k} is, at least in part, the result of inhibition of PI3K. Although we have found that PI3K also participates in PDGF-mediated activation of pp70^{S6k} (27), increasing intracellular cAMP does not dramatically inhibit activation of PI3K by PDGF (45) and under similar conditions has only a small effect on pp70^{S6k} (26). Thus, the exact mechanism of how elevated cAMP inhibits IL-2-regulated PI3K remains to be elucidated. Interestingly, the fact that PKA activation antagonizes IL-2-mediated activation of PI3K and has little effect on PDGF-, epidermal growth factor-, or insulin receptor-mediated activation of PI3K (14) may be a reflection of the different mechanisms of PI3K activation by these distinct receptor systems.

The characterization of the cAMP effect is important not only to enrich our knowledge of intracellular signal transduc-

tion but also because endogenous ligands, such as prostaglandin E2, which elevate cAMP may play a role in the physiologic inhibitory modulation of the immune response (57). Elevated cAMP levels are also linked to thymocyte apoptosis and may serve in induction of anergy (33, 70). With regard to IL-2-dependent T-cell proliferation, the eventual definition of the steps necessary for progression through G_1, and committal to cell division, should offer the potential to develop the means to therapeutically modulate immune responses.

Do distinct lipid kinases participate in the regulation of pp70^{S6k}? Recently, putative yeast and mammalian TORs have been isolated (13, 15, 52, 89, 90). On the basis of protein sequence data, these have been suggested to be related to mammalian and a second yeast PI3K, Vps34. However, a knockout of Vps34 completely abolishes yeast PI3K activity and production of phosphatidylinositol 3-phosphate, whereas there is no biochemical or genetic or biological evidence that the TORs are indeed PI3Ks (15, 52, 53, 62, 95). To determine if mammalian, cytokine-activated PI3K was inhibited by the T-cell immunosuppressant rapamycin, we measured PI3K activity in T cells incubated with IL-2, in the absence or presence of rapamycin, in antiphosphotyrosine immune complexes. Under these conditions, pp70^{S6k} activity was completely inhibited whereas PI3K activity was not inhibited either in rapamycin-treated cells or when rapamycin and FKBP12 were added to the in vitro assay. These results are consistent with our evidence that PI3K mediates IL-2-dependent activation of pp70^{S6k} and that the PI3K-specific inhibitor wortmannin and rapamycin block IL-2-dependent signal transduction to pp70^{S6k} at distinct points within this signalling system (Fig. 13).

Roles of PI3K and pp70^{S6k} in cell growth and metabolism. In addition to many reports linking PI3K activity with cell proliferation and transformation (21, 82), there is growing evidence that this enzyme is involved in multiple cellular functions. There is also ample evidence supporting a role for pp70^{S6k} in cell growth (16, 28, 34, 63, 65, 86). How these signalling enzymes are coupled is currently unclear. One of the PI3K cellular products, phosphatidylinositol-3,4,5-P_3, is proposed to be a good candidate for a second messenger with the potential to regulate signal transduction (3, 55, 99, 108). It has recently been shown to activate the phorbol ester-insensitive PKC ζ isoform (PKCζ) in vitro (80). Thus, it is possible that PKCζ mediates signalling from PI3K to pp70^{S6k}. This possibility is particularly intriguing since PKCζ is insensitive to phorbol ester-mediated down-regulation (reviewed in reference 80) and participates in the regulation of cell proliferation and *Xenopus* oocyte maturation (6, 32). The suggested role of PKCζ as an upstream regulator of pp70^{S6k} remains to be investigated. In addition, there are other phorbol ester-independent PKC isoforms that may be candidates for PI3K-regulated signal transducers (17). It is also conceivable that the protein kinase activity associated with PI3K (as opposed to its lipid kinase activity) (20, 64) may mediate activation of the pp70^{S6k} signalling cascade. Since wortmannin effectively inhibits both the lipid and protein kinase activity of PI3K (22, 64), we cannot presently hypothesize by which mechanism signal transmission occurs. Yet another possibility is that other signalling molecules bind to PI3K that has been recruited to the membrane by receptor binding and that this molecule(s) in turn is responsible for pp70^{S6k} activation. In this model, one would need to propose that binding of either wortmannin or LY294002 to the p110 catalytic subunit of PI3K would also antagonize its ability to interact with these other potential signal transducers. Now that we have established the basis for a direct molecular assay, these and other possibilities can be tested.

A role for PI3K in intracellular vesicular transport has been suggested (18, 60). In *Saccharomyces cerevisiae*, Vps34P, a putative homolog of the PI3K p110 catalytic subunit which possesses PI3K activity (95), is involved in the regulation of protein sorting through vesicular transport (53). Also, recent studies in mammalian cells using wortmannin indicate that PI3K participates in the regulation of histamine release in response to immunoglobulin E (117) and GLUT4 translocation to the plasma membrane in response to insulin (23, 58). Previous experiments indicate that rapamycin does not block either protein-sorting events (42, 110), thus excluding pp70^{S6k} from their regulation. These and our results are consistent with the pathway regulated by PI3K bifurcating at some point between the PI3K and the TOR. Alternatively, instead of participating in a linear pathway, PI3K and mammalian TOR may reside in parallel pathways that are both required for activation of pp70^{S6k}. It is clear that continued characterization of this signalling system will provide important information regarding the IL-2R signal transduction as well as the regulation of the T-cell cycle.

ACKNOWLEDGMENTS

We express sincere gratitude to L. C. Cantley, C. L. Carpenter, B. Drucker, B. C. Duckworth, M. K. Gately, C. W. Greene, and S. N. Seghal for providing various reagents, technical advice, and unpublished information. We also thank members of our laboratory for helpful comments.

This work was supported by Public Health Service grant CA46595 from the National Institute of Health, Harvard Medical School Funds for Discovery, and the Lucille P. Markey Charitable Trust. K.P.L. is a Howard Hughes Medical Institute Medical Student Research Training Fellow. M.M. was supported by a postdoctoral fellowship from the Ministerio de Educación y Ciencia (Spain). J.B. is an Established Investigator of the American Heart Association.

M.M. and K.P.L. made equal contributions to this work.

REFERENCES

1. **Alcorta, D. A., C. M. Crews, L. J. Sweet, L. Bankston, S. W. Jones, and R. L. Erikson.** 1989. Sequence and expression of chicken and mouse *rsk*: homologs of *Xenopus* ribosomal S6 kinase. Mol. Cell. Biol. **9:**3850–3859.
2. **Arcaro, A., and M. P. Wymann.** 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem. J. **296:**297–301.
3. **Auger, K. R., L. A. Serunian, S. P. Soltoff, P. Libby, and L. C. Cantley.** 1989. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell **57:**167–175.
4. **Augustine, J. A., S. L. Sutor, and R. T. Abraham.** 1991. Interleukin 2- and polyomavirus middle T antigen-induced modification of phosphatidylinositol 3-kinase activity in activated T lymphocytes. Mol. Cell. Biol. **11:**4431–4440.
5. **Banerjee, P., M. F. Ahmad, J. R. Grove, C. Kozlosky, D. J. Price, and J. Avruch.** 1990. Molecular structure of a major insulin/mitogen-activated 70-kDa S6 protein kinase. Proc. Natl. Acad. Sci. USA **87:**8550–8554.
6. **Berra, E., M. T. Diaz-Meco, I. Dominguez, M. M. Municio, L. Sanz, J. Lozano, R. S. Chapkin, and J. Moscat.** 1993. Protein kinase C ζ isoform is critical for mitogenic signal transduction. Cell **74:**555–563.
7. **Bierer, B. E., P. S. Mattila, R. F. Standaert, L. A. Herzenberg, S. J. Burakoff, G. Crabtree, and S. L. Schreiber.** 1990. Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. Proc. Natl. Acad. Sci. USA **87:**9231–9235.
8. **Blenis, J.** Unpublished data.
9. **Blenis, J.** 1993. Signal transduction via the MAP kinases: proceed at your own RSK. Proc. Natl. Acad. Sci. USA **90:**5889–5892.
10. **Blenis, J., J. Chung, E. Erikson, D. A. Alcorta, and R. L. Erikson.** 1991. Distinct mechanisms for the activation of the RSK kinases/MAP2 kinase/pp90^{rsk} and pp70-S6 kinase signaling systems are indicated by inhibition of protein synthesis. Cell. Growth Differ. **2:**279–285.
11. **Blenis, J., and R. L. Erikson.** 1986. Stimulation of ribosomal protein S6 kinase activity by pp60$^{v\text{-}src}$ or by serum: dissociation from phorbol ester-stimulated activity. Proc. Natl. Acad. Sci. USA **82:**1733–1737.
12. **Boguski, M. S., and F. McCormick.** 1993. Proteins regulating Ras and its relatives. Nature (London) **356:**643–654.
13. **Brown, E. J., M. W. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane, and S. L. Schreiber.** 1994. A mammalian protein targeted by G1-arresting

rapamycin-receptor complex. Nature (London) **369:**756–758.
14. **Burgering, B. M., G. J. Pronk, P. C. vanWeeren, P. Chardin, and J. L. Bos.** 1993. cAMP antagonizes p21^{ras}-directed activation of extracellular signal-regulated kinase 2 and phosphorylation of mSos nucleotide exchange factor. EMBO J. **12:**4211–4220.
15. **Cafferkey, R., P. R. Young, M. M. McLaughlin, D. J. Bergsma, Y. Koltin, G. Sathe, L. Faucette, W.-K. Eng, R. K. Johnson, and G. P. Livi.** 1993. Dominant missense mutations in novel yeast protein related to mammalian phosphatidylinositol 3-kinase and *VPS34* abrogate rapamycin cytotoxicity. Mol. Cell. Biol. **13:**6012–6023.
16. **Calvo, V., C. M. Crews, T. A. Vik, and B. E. Bierer.** 1992. Interleukin 2 stimulation of p70 S6 kinase activity is inhibited by the immunosuppressant rapamycin. Proc. Natl. Acad. Sci. USA **89:**7571–7575.
17. **Cantley, L.** 1994. Lipid second messengers. Cell **77:**329–334.
18. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64:** 281–302.
19. **Cantrell, D. A., M. Izquierdo, K. Reif, and M. Woodrow.** 1993. Regulation of the PtdIns-3-kinase and the guanine nucleotide binding protein p21^{ras} during signal transduction by the T cell antigen receptor and the interleukin-2 receptor. Semin. Immunol. **5:**319–326.
20. **Carpenter, C. L., K. R. Auger, B. C. Duckworth, W.-M. Hou, B. Schaffhausen, and L. C. Cantley.** 1993. A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. Mol. Cell. Biol. **13:**1657–1665.
21. **Carpenter, C. L., and L. C. Cantley.** 1990. Phosphoinositide kinases. Biochemistry **29:**11147–11156.
22. **Carpenter, C. L., B. C. Duckworth, and L. C. Cantley (Harvard University).** 1994. Personal communication.
23. **Cheatham, B.** 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.
24. **Chen, R.-H., and J. Blenis.** 1990. Identification of *Xenopus* S6 protein kinase homologs (pp90^{rsk}) in somatic cells: phosphorylation and activation during initiation of cell proliferation. Mol. Cell. Biol. **10:**3204–32015.
25. **Chen, R.-H., J. Chung, and J. Blenis.** 1991. Regulation of pp90^{rsk} phosphorylation and S6 phosphotransferase activity in Swiss 3T3 cells by growth factor-, phorbol ester-, and cyclic AMP-mediated signal transduction. Mol. Cell. Biol. **11:**1861–1867.
26. **Chung, J.** Unpublished data.
27. **Chung, J., T. C. Grammer, K. P. Lemon, A. Kazlauskas, and J. Blenis.** 1994. PDGF- and insulin-dependent pp70^{S6k} activation mediated by phosphatidylinositol-3-OH kinase. Nature (London) **370:**71–75.
28. **Chung, J., C. J. Kuo, G. R. Crabtree, and J. Blenis.** 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70kd S6 protein kinases. Cell **69:**1227–1236.
29. **Cook, S. J., and F. McCormick.** 1993. Inhibition by cAMP of Ras-dependent activation of Raf. Science **262:**1069–1072.
30. **Crews, C. M., and R. L. Erikson.** 1993. Extracellular signals and reversible protein phosphorylation: what to MEK of it all. Cell **74:**215–217.
31. **Davis, R. J.** 1993. The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. **268:**14553–14556.
32. **Dominguez, I., M. T. Diaz-Meco, M. M. Municio, E. Berra, A. Garcia de Herreros, M. E. Cornet, L. Sanz, and J. Moscat.** 1992. Evidence for a role of protein kinase C ζ subspecies in maturation of *Xenopus laevis* oocytes. Mol. Cell. Biol. **12:**3776–3783.
33. **Dowd, D. R., and R. L. Miesfeld.** 1992. Evidence that glucocorticoid- and cyclic AMP-induced apoptosis pathways in lymphocytes share distal events. Mol. Cell. Biol. **12:**3600–3608.
34. **Dumont, F. J., A. Altmeyer, C. Kastner, P. A. Fischer, K. P. Lemon, J. K. Chung, J. Blenis, and M. J. Staruch.** 1994. Relationship between multiple biologic effects of rapamycin and the inhibition of pp70 S6 protein kinase activity—analysis in mutant clones of a T-cell lymphoma. J. Immunol. **152:** 992–1003.
35. **Dumont, F. J., M. R. Melino, M. J. Staruch, S. L. Koprak, P. A. Fischer, and N. H. Sigal.** 1990. The immunosuppressive macrolides FK506 and rapamycin act as reciprocal antagonists in murine T cells. J. Immunol. **144:** 1418–1424.
36. **Erikson, R. L.** 1991. Structure, expression, and regulation of protein kinases involved in the phosphorylation of ribosomal protein S6. J. Biol. Chem. **266:** 6007–6010.
37. **Escobedo, J. A., S. Navankasattusas, W. M. Kavanaugh, D. Milfay, V. A. Fried, and L. T. Williams.** 1991. cDNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of PI3-kinase to the PDGFb-receptor. Cell **65:**75–82.
38. **Fairhurst, R. M., M. Daeipour, M. C. Amaral, and A. E. Nel.** 1993. Activation of mitogen-activated protein kinase/ERK-2 in phytohaemagglutinin blasts by recombinant interleukin-2; contrasting features with CD3 activation. Immunology **79:**112–118.
39. **Feig, L. A.** 1993. The many roads that lead to Ras. Science **260:**767–768.
40. **Ferrari, S., W. Bannwarth, S. J. Morley, N. F. Totty, and G. Thomas.** 1992. Activation of pp70^{S6k} is associated with phosphorylation of four clustered sites displaying Ser/Thr-Pro motifs. Proc. Natl. Acad. Sci. USA **89:**7282–7286.
41. **Ferrari, S., R. B. Pearson, M. Siegmann, S. C. Kozma, and G. Thomas.** 1993. The immunosuppressant rapamycin induces inactivation of p70^{s6k} through dephosphorylation of a novel set of sites. J. Biol. Chem. **268:**16091–16094.
42. **Fingar, D. C., S. F. Hausdorff, J. Blenis, and M. J. Birnbaum.** 1993. Dissociation of pp70 ribosomal protein S6 kinase from insulin-stimulated glucose transport in 3T3-L1 adipocytes. J. Biol. Chem. **268:**3005–3008.
43. **Flanagan, W. M., B. Corthesy, R. J. Bram, and G. R. Crabtree.** 1991. Nuclear association of a T-cell transcription factor blocked by FK506 and cyclosporin A. Nature (London) **352:**803–807.
44. **Frodin, M., P. Peraldi, and E. Van Obberghen.** 1994. Cyclic AMP activates the mitogen-activated protein kinase cascade in PC12 cells. J. Biol. Chem. **269:**6207–6214.
45. **Grammer, T. C.** Unpublished data.
46. **Graves, L. M., K. E. Bornfeldt, E. W. Raines, B. C. Potts, S. G. Macdonald, R. Ross, and E. G. Krebs.** 1993. Protein kinase A antagonizes platelet-derived growth factor-induced signaling by mitogen-activated protein kinase in human arterial smooth muscle cells. Proc. Natl. Acad. Sci. USA **90:** 10300–10304.
47. **Grove, J. R., P. Banerjee, A. Balasubramanyam, P. J. Coffer, D. J. Price, J. Avruch, and J. R. Woodgett.** 1991. Cloning and expression of two human p70 S6 kinase polypeptides differing only at their amino termini. Mol. Cell. Biol. **11:**5541–5550.
48. **Gulbins, E., K. M. Coggeshall, G. Baier, D. Telford, C. Langlet, G. Baier-Bitterlich, N. Bonnefoy-Berard, P. Burn, A. Wittinghofer, and A. Altman.** 1994. Direct stimulation of Vav guanine nucleotide exchange activity for Ras by phorbol esters and diglycerides. Mol. Cell. Biol. **14:**4749–4758.
49. **Harding, M. W., A. Galat, D. E. Uehling, and S. L. Schreiber.** 1989. A receptor for the immunosuppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. Nature (London) **341:**758–760.
50. **Hatakeyama, M., T. Kono, N. Kobayashi, A. Kawahara, S. D. Levin, R. M. Perlmutter, and T. Taniguchi.** 1991. Interaction of the IL-2 receptor with the src-family kinase p56^{lck}: identification of a novel intermolecular association. Science **252:**1523–1528.
51. **Hatakeyama, M., H. Mori, T. Doi, and T. Taniguchi.** 1989. A restricted cytoplasmic region of IL-2 receptor β chain is essential for growth signal transduction but not for ligand binding and internalization. Cell **59:**837–845.
52. **Helliwell, S. B., P. Wagner, J. Kunz, M. Deuter-Reinhard, R. Henriquez, and M. Hall.** 1994. TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologs in yeast. Mol. Biol. Cell **5:**105–118.
53. **Herman, P. K., and S. D. Emr.** 1990. Characterization of VPS34, a gene requires for vacuolar protein sorting and vacuole segregation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**6742–6754.
54. **Hiles, I. D., M. Otsu, S. Volinia, M. J. Fry, I. Bout, R. Dhand, G. Panayotou, F. Ruiz-Larrea, A. Thompson, N. F. Totty, J. J. Hsuan, S. A. Courtneidge, and M. D. Waterfield.** 1992. Phosphatidylinositol 3-kinase: structure and expression of the 110 kD catalytic subunit. Cell **70:**419–429.
55. **Jackson, T. R., L. R. Stephens, and P. T. Hawkins.** 1992. Receptor specificity of growth factor-stimulated synthesis of 3-phosphorylated inositol lipids in Swiss 3T3 cells. J. Biol. Chem. **267:**16627–16636.
56. **Johnson, K. W., B. H. Davis, and K. A. Smith.** 1988. cAMP antagonizes interleukin 2-promoted T-cell cycle progression at a discrete point in early G1. Proc. Natl. Acad. Sci. USA **85:**6072–6076.
57. **Kammer, G. M.** 1988. The adenylate cyclase-cAMP-protein kinase A pathway and regulation of the immune response. Immunol. Today **9:**222–229.
58. **Kanai, F., K. Ito, M. Todaka, H. Hayashi, S. Kamohara, K. Ishii, T. Okada, O. Hazeki, M. Ui, and Y. Ebina.** 1993. Insulin-stimulated GLUT4 translocation is relevant to the phosphorylation of IRS-1 and the activity of PI3-kinase. Biochem. Biophys. Res. Commun. **195:**762–768.
59. **Karnitz, L. M., S. L. Sutor, and R. T. Abraham.** 1994. The Src-family kinase, Fyn, regulates the activation of phosphatidylinositol 3-kinase in an interleukin 2-responsive T cell line. J. Exp. Med. **179:**1799–1808.
60. **Kelly, K. L., N. B. Ruderman, and K. S. Chen.** 1992. Phosphatidylinositol-3-kinase in isolated rat adipocytes. J. Biol. Chem. **267:**3423–3428.
61. **Kolb, J. P., A. Abadie, A. Paul-Eugene, and B. Dugas.** 1993. Intracellular signaling events associated with the induction of DNA synthesis in human B lymphocytes. II. Different pathways triggered by IL-2 and IL-4. Cell Immunol. **146:**131–146.
62. **Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N. R. Movva, and M. N. Hall.** 1993. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell **73:**585–596.
63. **Kuo, C. J., J. Chung, D. F. Fiorentino, W. M. Flanagan, J. Blenis, and G. R. Crabtree.** 1992. Inhibition of IL-2 induced p70 S6 kinase activity by the immunosuppresive agent rapamycin. Nature (London) **358:**70–74.
64. **Lam, K., C. L. Carpenter, N. B. Ruderman, J. C. Friel, and K. L. Kelly.** 1994. The phosphatidylinositol 3-kinase serine kinase phosphorylates IRS-1. Stimulation by insulin and inhibition by wortmannin. J. Biol. Chem. **269:**20648–20652.
65. **Lane, H. A., A. Fernandez, N. J. C. Lamb, and G. Thomas.** 1993. p70^{S6k}

function is essential for G1 progression. Nature (London) **363:**170–172.
66. **Lingk, D. S., M. A. Chan, and E. W. Gelfand.** 1990. Increased cyclic adenosine monophosphate levels block progression by not initiation of human T cell proliferation. J. Immunol. **145:**449–455.
67. **Liu, J., J. D. Farmer, Jr., W. S. Lane, J. Friedman, I. Weissman, and S. L. Schreiber.** 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell **66:**807–815.
68. **Marshall, C. J.** 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Gen. Dev. **4:**82–89.
69. **Matten, W., I. Daar, and G. F. Vande Woude.** 1994. Protein kinase A acts at multiple points to inhibit *Xenopus* oocyte maturation. Mol. Cell. Biol. **14:**4419–4426.
70. **McConkey, D. J., S. Orrenius, and M. Jondal.** 1990. Agents that elevate cAMP stimulate DNA fragmentation in thymocytes. J. Immunol. **145:**1227–1230.
71. **Merida, I., E. Diez, and G. N. Gaulton.** 1991. IL-2 binding activates a tyrosine-phosphorylated phosphatidylinositol-3 kinase. J. Immunol. **147:** 2202–2207.
72. **Merida, I., P. Williamson, W. A. Kuziel, W. C. Greene, and G. N. Gaulton.** 1993. The serine-rich domain of the interleukin-2 receptor β chain is essential for interleukin-2-dependent tyrosine protein kinase and phosphatidylinositol-3-kinase activation. J. Biol. Chem. **268:**6765–6770.
73. **Mills, G. B., P. Girard, S. Grinstein, and E. W. Gelfand.** 1988. Interleukin-2 induces proliferation of T lymphocyte mutants lacking protein kinase C. Cell **55:**91–100.
74. **Mills, G. B., D. J. Stewart, A. Mellors, and E. W. Gelfand.** 1986. Interleukin-2 does not induce phosphatidylinositol hydrolysis in activated T cells. J. Immunol. **136:**3019–3024.
75. **Minami, Y., T. Kono, K. Yamada, and T. Taniguchi.** 1992. The interleukin-2 receptors: insights into a complex signalling mechanism. Biochim. Biophys. Acta **1114:**163–177.
76. **Monfar, M., and J. Blenis.** Unpublished data.
77. **Mukhopadhyay, N. K., D. J. Price, J. M. Kyriakis, S. Pelech, J. Sanghera, and J. Avruch.** 1992. An array of insulin-activated, proline-directed serine/threonine protein kinases phosphorylate the p70 S6 kinase. J. Biol. Chem. **267:**3325–3335.
78. **Nakanishi, H. N., K. A. Brewer, and J. H. Exton.** 1993. Activation of the ζ isozyme of protein kinase C by phosphatidylinositol 3,4,5-triphosphate. J. Biol. Chem. **268:**13–16.
79. **Nishizuka, Y.** 1983. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Philos. Trans. R. Soc. Lond. B **302:**101–102.
80. **Nishizuka, Y.** 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science **258:**607–614.
81. **Otsu, M., I. Hiles, I. Gout, M. J. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, A. D. Smith, S. J. Morgan, S. A. Courtneidge, P. J. Parker, and M. D. Waterfield.** 1991. Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60$^{c\text{-}src}$ complexes, and PI3-kinase. Cell **65:**91–104.
82. **Parker, P. J., and M. D. Waterfield.** 1992. Phosphatidylinositol 3-kinase: a novel effector. Cell Growth Differ. **3:**747–752.
83. **Pawson, T., and G. D. Gish.** 1992. SH2 and SH3 domains: from structure to function. Cell **71:**359–362.
84. **Pelech, S. L., and E. G. Krebs.** 1987. Mitogen-activated S6 kinase is stimulated via protein kinase C-dependent and -independent pathways in Swiss 3T3 cells. J. Biol. Chem. **262:**11598–11606.
85. **Price, D. J., J. R. Grove, V. Calvo, J. Avruch, and B. E. Bierer.** 1992. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. Science **257:**973–977.
86. **Reinhard, C., A. Fernandez, N. J. C. Lamb, and G. Thomas.** 1994. Nuclear localization of p85^{S6k}; functional requirement for entry into S phase. EMBO J. **13:**1557–1565.
87. **Remillard, B., R. Petrillo, W. Maslinski, M. Tsudo, T. B. Strom, L. C. Cantley, and L. Varticovski.** 1991. Interleukin-2 receptor regulates activation of phosphatidylinositol 3-kinase. J. Biol. Chem. **266:**14167–14170.
88. **Roskoski, R. J.** 1983. Assays of protein kinase. Methods Enzymol. **99:**3–7.
89. **Sabatini, D. M., H. Erdjument-Bromage, M. Lui, P. Tempst, and S. H. Snyder.** 1994. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell **78:** 35–43.
90. **Sabers, C. J., G. Wiederrecht, J. M. Williams, M. M. Martin, F. J. Dumont, and R. T. Abraham.** Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. J. Biol. Chem., in press.
91. **Sawami, H., N. Terada, R. A. Franklin, H. Okawa, T. Uchiyama, J. J. Lucas, and E. W. Gelfland.** 1992. Signal transduction by interleukin 2 in human T cells: activation of tyrosine and ribosomal S6 kinases and cell-cycle regulatory genes. J. Cell. Physiol. **151:**367–377.
92. **Schreiber, S. L.** 1991. Chemistry and biology of the immunophilins and signaling pathways. Science **251:**283–287.
93. **Schreiber, S. L.** 1992. Immunophilin-sensitive protein phosphatase action in cell signaling pathways. Cell **70:**365–368.
94. **Schreiber, S. L., and G. Crabtree.** 1992. The mechanism of action of cyclosporin A and FK506. Immunol. Today **13:**136–142.
95. **Schu, P. V., K. Takegawa, M. J. Fry, J. H. Stack, M. D. Waterfield, and S. D. Emr.** 1993. Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. Science **260:**88–91.
96. **Sevetson, B. R., X. Kong, and J. C. Lawrence, Jr.** 1993. Increasing cAMP attenuates activation of mitogen-activated protein kinase. Proc. Natl. Acad. Sci. USA **90:**10305–10309.
97. **Siekierka, J. J., S. H. Y. Hung, M. Poe, C. S. Lin, and N. H. Sigal.** 1989. A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature (London) **341:**755–757.
98. **Skolnik, E. Y., B. Margolis, M. Mohammadi, L. E. R. Fischer, A. Drepps, A. Ulrich, and J. Schlessinger.** 1991. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell **65:**83–90.
99. **Stephens, L. R., K. T. Hughes, and R. F. Irvine.** 1991. Pathway of phosphatidylinositol (3,4,5)-triphosphate synthesis in activated neutrophils. Nature (London) **351:**33–39.
100. **Stewart, M. J., and R. Denell.** 1993. Mutations in the *Drosophila* gene encoding ribosomal protein S6 cause tissue overgrowth. Mol. Cell. Biol. **13:** 2524–2535.
101. **Susa, M., S. Vulevic, H. Lane, and G. Thomas.** 1992. Inhibition or down-regulation of protein kinase C attenuates late phase p70^{S6k} activation induced by epidermal growth factor but not by platelet-derived growth factor or insulin. J. Biol. Chem. **267:**6905–6909.
102. **Taichman, R., I. Merida, T. Torigoe, G. N. Baulton, and J. C. Reed.** 1993. Evidence that protein tyrosine kinase p56-Lck regulates the activity of phosphatidylinositol-3′-kinase in interleukin-2-dependent cells. J. Biol. Chem. **268:**20031–20036.
103. **Taniguchi, T., and Y. Minami.** 1993. The IL-2/IL-2 receptor system: a current overview. Cell **73:**5–8.
104. **Taylor, S. S., J. A. Buechler, and W. Yonemoto.** 1990. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu. Rev. Biochem. **59:**971–1005.
105. **Taylor, S. S., D. R. Knighton, J. Zheng, J. M. Sowadski, C. S. Gibbs, and M. J. Zoller.** 1993. A template for the protein kinase family. Trends Biochem. Sci. **18:**84–89.
106. **Terada, N., J. J. Lucas, A. Szepesi, R. A. Franklin, K. Takase, and E. W. Gelfand.** 1992. Rapamycin inhibits the phosphorylation of p70 S6 kinase in IL-2 and mitogen-activated human T cells. Biochem. Biophys. Res. Commun. **186:**1315–1321.
107. **Terao, K., and K. Ogata.** 1970. Preparation and some properties of active subunits from rat liver ribosomes. Biochem. Biophys. Res. Commun. **38:** 1315–1321.
108. **Traynor-Kaplan, A. E., A. L. Harris, B. L. Thompson, P. Taylor, and L. Sklar.** 1988. An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. Nature (London) **334:**353–356.
109. **Trouche, D., P. Robin, D. Robillard, P. Sassone-Corsi, and A. Harel-Bellan.** 1991. c-fos transcriptional activation by IL-2 in mouse CTL-L2 cells is mediated through two distinct signal transduction pathways converging in the same enhancer element. J. Immunol. **147:**2398–2403.
110. **Tsai, M., R.-H. Chen, S.-Y. Tam, J. Blenis, and S. J. Galli.** 1993. Activation of MAP kinases, pp90^{rsk} and pp70-S6 kinases in mouse mast cells by signaling through the c-*kit* receptor tyrosine kinase or Fc$_\varepsilon$RI: rapamycin inhibits activation of pp70-S6 kinase and proliferation in mouse mast cells. Eur. J. Immunol. **23:**3286–3291.
111. **Valge, V. E., J. G. P. Wong, B. M. Datlof, A. J. Sinskey, and A. Rao.** 1988. Protein kinase C is required for responses to T cell receptor ligands but not to interleukin-2 in T cells. Cell **55:**101–112.
112. **Vlahos, C. J., W. F. Matter, K. Y. Hui, and R. F. Brown.** 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.
113. **Watson, K. L., K. D. Konrad, D. F. Woods, and P. J. Bryant.** 1992. *Drosophila* homolog of the human S6 ribosomal protein is required for tumor suppression in the hematopoietic system. Proc. Natl. Acad. Sci. USA **89:** 11302–11306.
114. **Whisler, R. L., L. Beiqing, I. S. Grants, and Y. G. Newhous.** 1992. Cyclic AMP modulation of human B cell proliferative responses: role of cAMP-dependent protein kinases in enhancing B cell responses to phorbol diesters and ionomycin. Cell. Immunol. **142:**398–415.
115. **Whitman, M., D. R. Kaplan, B. Schaffhausen, L. Cantley, and T. M. Roberts.** 1985. Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. Nature (London) **315:**239–242.
116. **Wu, J., P. Dent, T. Jelinek, A. Wolfman, M. J. Weber, and T. W. Sturgill.** 1993. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3′,5′-monophosphate. Science **262:**1065–1069.
117. **Yano, H., S. Nakanishi, K. Kimura, N. Hanai, Y. Saitoh, Y. Fukui, Y. Nomomura, and Y. Matsuda.** 1993. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J. Biol. Chem. **268:**25846–25856.