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Interleukin-2 Triggers a Novel Phosphatidylinositol 3-Kinase-Dependent MEK Activation Pathway

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Phosphatidylinositol 3-kinase (PI3-K) has been implicated as a signal-transducing component in interleukin-2 (IL-2)-induced mitogenesis. However, the function of this lipid kinase in regulating IL-2-triggered downstream events has remained obscure. Using the potent and specific PI3-K inhibitor, wortmannin, we assessed the role of PI3-K in IL-2-mediated signaling and proliferation in the murine T-cell line CTLL-2. Addition of the drug to exponentially growing cells resulted in an accumulation of cells in the G_0/G_1 phase of the cell cycle. Furthermore, wortmannin also partially suppressed IL-2-induced S-phase entry in G_1-synchronized cells. Analysis of IL-2-triggered signaling pathways revealed that wortmannin pretreatment resulted in complete inhibition of IL-2-provoked p70 S6 kinase activation and also attenuated IL-2-induced MAP kinase activation at drug concentrations identical to those required for inhibition of PI3-K catalytic activity. Wortmannin also diminished the IL-2-triggered activation of the MAP kinase activator, MEK, but did not inhibit activation of Raf, the canonical upstream activator of MEK. These results suggest that a novel wortmannin-sensitive activation pathway regulates MEK and MAP kinase in IL-2-stimulated T lymphocytes.

The interaction of a resting T cell with appropriately presented antigen initiates cell cycle entry (G_0- to G_1-phase transition) and the expression of high-affinity interleukin-2 (IL-2) receptors (IL-2R). The binding of IL-2 to the IL-2R then elicits G_1- to S-phase progression and commits the cell to traverse the remainder of the cell cycle. The high-affinity IL-2R is minimally composed of an IL-2Rα (p55), IL-2Rβ (p70), and γ_c (p64) subunit (57). However, heterodimerization of the IL-2Rβ and γ_c intracellular domains is sufficient to trigger the mitogenic response (42, 44).

Although the IL-2R subunits exhibit no intrinsic protein tyrosine kinase (PTK) activity, IL-2-induced PTK activation is requisite for proliferation (47). The IL-2Rβ chain is physically and functionally coupled to the Src-family PTKs, Lck (20, 21), Fyn (25, 26, 36), and Lyn (59). Additionally, the newly discovered JAK family PTKs, JAK1 and JAK3, are also activated by IL-2 (22, 65). Ligand-induced PTK activation elicits the downstream activation of multiple signaling components, one of which is the well-defined Ras/MAP kinase pathway. The Ras/MAP kinase pathway comprises a set of ubiquitous and highly conserved molecules that orchestrate the delivery of signals from the cell membrane to the nucleus (reviewed in reference 34). IL-2R ligation appears to regulate Ras via the tyrosine phosphorylation of the adaptor protein, Shc (8, 50, 70). Tyrosine-phosphorylated Shc then associates with the Src homology 2 (SH2) domain of the adaptor protein, Grb2 (2, 70), which is constitutively bound to the mammalian homolog of the Ras guanine-nucleotide exchange protein, Son of Sevenless (SOS) (18, 31). Complex formation results in the SOS-catalyzed exchange of GDP for GTP on Ras, with the concomitant activation of Ras (17, 54). Activated Ras interacts with the regulatory N terminus of the serine-threonine kinase, Raf (38, 62, 64, 68), localizing Raf to the membrane and providing a requisite signal for Raf activation (30, 55). Activated Raf then initiates a protein kinase cascade by phosphorylating and activating MEK (33), which, in turn, phosphorylates and activates MAP kinase (14, 33, 43).

The studies cited above define a linear signal transduction pathway leading to MAP kinase activation. However, recent data suggest that other signaling events also regulate the activity of this signaling cascade (7, 28, 67, 69). In addition to activating the Ras pathway, IL-2 also activates phosphatidylinositol 3-kinase (PI3-K) (4, 35, 51) via a Src family kinase-dependent mechanism (25). This heterodimeric lipid kinase comprises an 85-kDa regulatory subunit and a 110-kDa catalytic subunit, which phosphorylate phosphatidylinositol at the D-3 hydroxyl of the inositol ring (reviewed in reference 15). PI3-K activation correlates closely with tyrosine kinase growth factor receptor-induced proliferation (60) and with the transforming potential of polyomavirus middle-sized T antigen (32). Although PI3-K is ubiquitously expressed and activated by nearly all PTK-dependent receptors, the role of PI3-K in both PTK-dependent receptor-regulated proliferation and signal propagation is currently unclear.

To address the role of PI3-K in IL-2-provoked signal transduction and proliferation in T lymphocytes, we utilized wortmannin, a pharmacologic inhibitor of PI3-K (45, 46, 49, 58). Like the immunosuppressive drug rapamycin, which inhibits IL-2-induced p70 S6 kinase ($pp70^{S6K}$) activation (9, 13, 27), wortmannin also ablated IL-2-triggered $pp70^{S6K}$ activation. Additionally, wortmannin partially suppressed IL-2-provoked MAP kinase activation, and yet the drug had no effect on phorbol ester-induced activation of MAP kinase. Parallel studies revealed that wortmannin also inhibited the IL-2-induced activation of the upstream MAP kinase activator, MEK. Surprisingly, the drug did not impair the IL-2-provoked activation of Raf, an upstream activator of MEK. Taken in aggregate, these results suggest that inhibition of PI3-K function interferes with IL-2-dependent MAP kinase activation at the level of the upstream regulatory enzyme, MEK.

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MATERIALS AND METHODS

Cell culture. CTLL-2 cells were obtained from the American Type Culture Collection (Rockville, Md.) and were cultured as described previously (24). For experiments requiring growth factor-deprived cells, cells were cultured for 4 to 6 h as described previously (25). Treatment of cells with wortmannin was performed in the dark for 30 min prior to experimental manipulations. Clonal CD8-positive human T cells were isolated and propagated as described previously (63) and were a generous gift of P. J. Leibson (Mayo Clinic, Rochester, Minn.).

Reagents. Wortmannin was obtained from Sigma (St. Louis, Mo.). Stock solutions (1.2 mM) of wortmannin were prepared in dimethyl sulfoxide and stored at −70°C. The stock solutions were diluted directly into medium immediately prior to addition to cells. The final dimethyl sulfoxide concentrations to which cells were exposed did not exceed 0.025%. LY294002 (61) was generously provided by C. Vlahos (Lilly Research Laboratories, Indianapolis, Ind.). Stock solutions of LY294002 were prepared in dimethyl sulfoxide prior to dilution into medium. Radioiodinated IL-2 was from Amersham (Arlington Heights, Ill.). Human recombinant IL-2 was a generous gift of Hoffmann-LaRoche (Nutley, N.J.). MAP kinase substrate peptide, antiphosphotyrosine (pY) and anti-Grb2 monoclonal antibodies, and the anti-Shc and anti-Sos rabbit antisera were from Upstate Biotechnology, Inc. (Lake Placid, N.Y.). Rapamycin was a generous gift of Merck, Sharpe and Dohme (Rahway, N.J.). The anti-p85 antiserum has been described previously (6). Anti-Raf rabbit polyclonal immunoglobulin G (C-12) was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). The anti-MAP kinase rabbit antiserum (66) and the anti-pp70^{S6K} rabbit antiserum (13) have been previously described. The MEK cDNA was a generous gift from G. Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.). Herbimycin A and okadaic acid were from LC Laboratories (Woburn, Mass.).

Quantitation of DNA synthesis and cell cycle analysis. DNA synthesis was quantitated as described previously (1), except that CTLL-2 cells (10^4 per well) were deprived of growth factor for 12 h and pretreated with the indicated concentrations of drug at 37°C for 30 min prior to addition of IL-2. After 12 h, [^{3}H]thymidine was added and the cells were incubated for an additional 6 h. Cell cycle analysis was performed as described previously (39), and cell cycle distributions were determined with Modfit software (Verity, Inc., Topsham, Maine).

PI3-K assays. Exponentially growing CTLL-2 cells (10^7 per sample) were washed once with RPMI 1640 containing 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 7.2), and 10% fetal bovine serum; were resuspended at 10^7/ml in the same medium; and were treated with the indicated concentrations of wortmannin in the dark at 37°C for 30 min. Cells were then centrifuged and lysed in PI3-K lysis buffer (25). Cleared lysates were incubated with protein A-conjugated Sepharose (Sigma) and 10 μl of rabbit anti-p85 antiserum for 1 h. After washing, immunoprecipitated lipid kinase activity was assayed and quantitated as described previously (25).

IL-2 internalization. Exponentially growing CTLL-2 cells (10^6 per time point) were stripped of bound IL-2 with a low-pH wash as described previously (24) and were pretreated with medium or 100 nM wortmannin at 37°C for 30 min. ^{125}I-IL-2 internalization was quantitated as described previously (52).

MAP kinase assays. MAP kinase activity was assayed by a modification of previously reported procedures (3, 56). Growth factor-deprived CTLL-2 cells (5×10^6 per sample) were pretreated with drug and stimulated as outlined in each experiment. At each time point, cells were pelleted in a microcentrifuge and were washed with ice-cold phosphate-buffered saline (PBS) (10 mM sodium phosphate, 150 mM NaCl [pH 7.4]). One milliliter of lysis buffer [25 mM Tris (pH 7.4), 25 mM NaCl, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithiothreitol, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 40 mM 4-nitrophenyl phosphate, 30 mM β-glycerophosphate, 10 μg of leupeptin per ml, 5 μg of pepstatin per ml, and 5 μg of aprotinin per ml) was added, and the samples were sonicated with three 5-s bursts interspersed with 30 s of cooling on ice. Insoluble material was removed by centrifugation (5 min, 15,000 × *g*). The resulting supernatants were mixed with 50 μl of packed phenyl-Sepharose (Sigma), and 110 μl of ethylene glycol was added to each sample. Prior to use, the phenyl-Sepharose was washed twice with lysis buffer. Samples were rotated at 4°C for 10 min, and the resin was washed twice with lysis buffer containing 10% ethylene glycol (without protease inhibitors) and twice with lysis buffer containing 35% ethylene glycol. MAP kinase was eluted with 150 μl of lysis buffer containing 60% ethylene glycol. The eluate (10 μl) was added to 20 μl of kinase assay mixture containing 12.5 mM 3-(*N*-morpholino) propanesulfonic acid, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.05 mM NaF (pH 7.4), 1 mg of myelin basic protein phosphoacceptor peptide per ml (Upstate Biotechnology Inc., amino acids 95 to 98), 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP (4,500 Ci/mmol) per reaction. The kinase reaction was performed at 23°C for 10 min and was terminated by spotting on Whatman P81 paper (Maidstone, England). The papers were washed once in 1% H_3PO_4 containing 15 mM $Na_4P_2O_7$ for 15 min and then three times for 15 min each in 1% H_3PO_4. Paper-bound ^{32}P was quantitated by liquid scintillation counting. MAP kinase was also assayed in anti-MAP kinase immunoprecipitates as previously described (66).

Immunoblotting. Anti-pY immunoblots were performed as described previously (25). All other immunoblots were blocked in 50 mM Tris (pH 7.4)–150 mM NaCl–0.2% Tween 20 (TBST) containing either 2% nonfat dried milk (for MAP kinase) or 1% bovine serum albumin (for Shc, Grb2, and Sos) and were then incubated with a 1:5,000 dilution (anti-MAP kinase), a 1:2,000 dilution (anti-Shc), or a 1:1,000 dilution (anti-Grb2 and anti-Sos) in TBST containing 0.1% bovine serum albumin. The blots were washed and incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated protein A (Amersham) and were developed with enhanced chemiluminescence reagents (Amersham). For the Grb2 immunoblots, the membranes were washed and incubated with a rabbit anti-mouse secondary antibody prior to incubation with horseradish peroxidase-conjugated protein A.

Ras assays. CTLL-2 cells (10^7 per sample) were deprived of growth factor and were incubated in phosphate-free RPMI medium containing 1% bovine serum albumin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10 mM HEPES (pH 7.2), and 0.2 mCi of [^{32}P]P_i per ml for 3 h. Where indicated, cells were pretreated with 9 μM herbimycin A for 1 h in the same medium without 2-mercaptoethanol. Herbimycin A-treated cells were labeled for 3 h with 0.2 mCi of [^{32}P]P_i per ml. Thirty minutes prior to stimulation, 75 μM Na_3VO_4 was added to all samples and 100 nM wortmannin was added to the indicated samples. Cells were stimulated with either 100 U of IL-2 per ml for 1, 2, or 5 min or with 100 ng of phorbol 12-myristate 13-acetate (PMA) per ml for 10 min. Stimulations were terminated with 30 ml of ice-cold PBS containing 0.5 mM EDTA. Cells were pelleted by centrifugation and lysed in 0.5 ml of lysis buffer (50 mM HEPES [pH 7.4], 100 mM NaCl, 5 mM $MgCl_2$, 1 mg of bovine serum albumin per ml, 1% Triton X-100, 10 μg of leupeptin per ml, 5 μg of pepstatin per ml, 5 μg of aprotinin per ml). The samples were diluted with 0.5 ml of lysis buffer containing 1 M NaCl, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), and 5 μg of anti-Ras monoclonal antibodies (Y13-259 [19]) was added. The immune complexes were recovered with 15 μl of packed protein A-Sepharose prebound with 5 μg of rabbit anti-rat immunoglobulin G. Samples were rotated at 4°C for 90 min and were washed eight times with 50 mM HEPES (pH 7.4)–500 mM NaCl–5 mM $MgCl_2$–0.1% Triton X-100–0.005% SDS. Ras-bound nucleotides were eluted in 0.2% SDS–2 mM EDTA–2 mM dithiothreitol–0.25 mM GDP–0.25 mM GTP at 68°C for 20 min. Eluates were adjusted to 1 mM GTP and 1 mM GDP and were applied to polyethyleneimine cellulose thin-layer chromatography plates. After drying, the plates were dipped in methanol-H_2O (1:1), were air dried briefly, and were developed in running buffer containing 4.8 M ammonium formate and 0.8 M HCl. Radioactivity was quantitated with an Ambis imaging system.

MEK, Raf, and pp70^{S6K} kinase assays. MEK catalytic activity was assayed with a kinase-dead glutathione-*S*-transferase (GST)–ERK1 fusion protein as a substrate. Growth factor-deprived CTLL-2 cells (5×10^6 per sample) were pretreated with drug as indicated and were then stimulated with IL-2 for the indicated times. Cells were centrifuged, and the cell pellet was resuspended in lysis buffer (20 mM Tris, 40 mM $Na_2P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 10 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate, 40 mM β-glycerophosphate [pH 8.0], 40 mM 4-nitrophenylphosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 20 μg of leupeptin, pepstatin A, and aprotinin per ml, 0.6 μM okadaic acid, 0.5 mM phosphoserine, 0.5 mM phosphotyrosine, and 1 mM phosphothreonine). SDS was added to the cleared lysates to a final concentration of 0.1%. MEK was then immunoprecipitated for 1 h with 3 μl of anti-MEK antiserum and protein A-Sepharose. Immunoprecipitates were washed three times with lysis buffer (without okadaic acid or phosphoamino acids) containing 0.1% SDS, three times with wash buffer (50 mM Tris [pH 7.5], 5 mM octyl-β-glucopyranoside, 1 mM dithiothreitol, 40 mM β-glycerophosphate), and twice with kinase buffer (40 mM Tris [pH 7.5], 80 mM NaCl, 8 mM $MgCl_2$, 1 mM EGTA, 5 mM octyl-β-glucopyranoside). Kinase reactions were initiated with 20 μl of kinase reaction mixture (kinase buffer containing 50 μg of kinase-dead GST-ERK1 per ml and 10 μCi of [γ-^{32}P]ATP per reaction). Samples were incubated at 30°C for 20 min, and the reactions were terminated with 20 μl of 4× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (40 mM Tris-HCl [pH 8.0], 40% glycerol, 20% 2-mercaptoethanol, 4% SDS, 4 mM EDTA, 0.01% bromophenol blue). Solubilized proteins were fractionated by SDS-PAGE (10% polyacrylamide gel) and were electrophoretically transferred to Immobilon-P (Millipore, Bedford, Mass.). Membrane-bound radioactivity was quantitated with an Ambis imaging system.

For Raf kinase assays, growth factor-deprived CTLL-2 cells were pretreated with medium or 100 nM wortmannin and stimulated with IL-2 or PMA for 5 min. The samples were prepared and kinase assays were performed as described above for MEK, with the following exceptions. Immunoprecipitates were prepared with 0.6 μg of anti-Raf antibody per sample. The substrate for the Raf assays was 1 μg of kinase-dead GST-MEK.

For pp70^{S6K} assays, CTLL-2 cells were deprived of growth factors for 4 h and restimulated with IL-2 or PMA for 20 min. Cells were lysed and pp70^{S6K} was immunopurified as described previously (41).

RESULTS

Wortmannin inhibits PI3-K in intact T cells. The availability of wortmannin, a potent and specific PI3-K inhibitor, allowed us to assess the role of PI3-K in IL-2-induced proliferation and

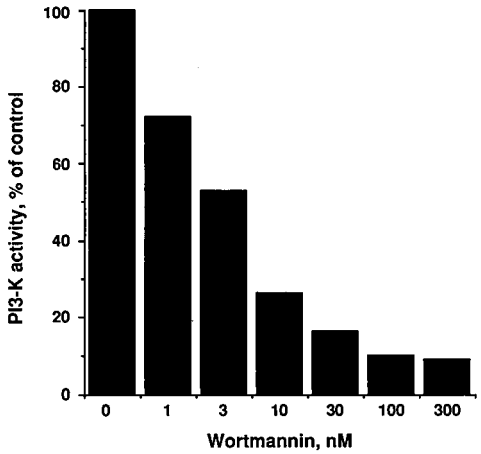

FIG. 1. Wortmannin inhibition of PI3-K. Exponentially growing CTLL-2 cells were pretreated with medium only or the indicated concentrations of wortmannin at 37°C for 30 min. Detergent lysates were immunoprecipitated with anti-p85 antiserum, and immunopurified PI3-K activity was assayed. Reaction products were separated by thin-layer chromatography. Radioactivity on thin-layer chromatography plates was quantitated with an Ambis imaging system. Bars represent the averages of three independent determinations.

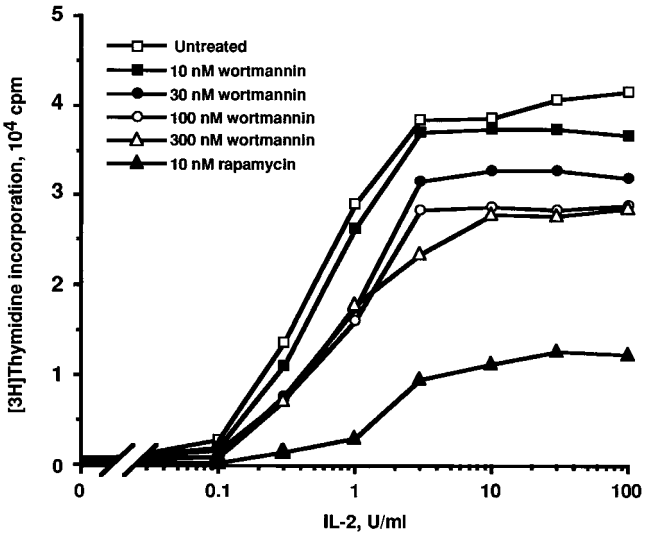

FIG. 2. IL-2-induced DNA synthesis in rapamycin- and wortmannin-pretreated cells. Cells were deprived of growth factors for 12 h and were pretreated with medium only or the indicated concentrations of either wortmannin or rapamycin for 30 min. Cells were then cultured with the indicated concentrations of IL-2 for 12 h. Radiolabeled [^{3}H]thymidine was added, and the samples were cultured for an additional 6 h. Similar results were observed in three independent experiments. Datum points represent the means of triplicate values. The coefficients of variation were less than 10%.

signal transduction events. To corroborate that wortmannin inhibits PI3-K in intact T cells, we treated exponentially growing CTLL-2 cells with the indicated concentrations of wortmannin for 30 min (Fig. 1). PI3-K was immunoprecipitated from detergent lysates with anti-p85 antiserum, and the lipid kinase activity of the immunopurified PI3-K was quantitated. The wortmannin concentration required for a 50% inhibition of immunoprecipitable PI3-K activity was approximately 4 nM, which agrees closely with the 50% inhibitory concentrations reported for other cell types (45, 46, 49, 58). Treatment of cells with 100 nM wortmannin resulted in maximal inhibition of p85-associated PI3-K activity.

Wortmannin's effect on IL-2-induced cell cycle progression and S-phase entry. Like many other mitogens, IL-2 stimulates PI3-K activity (4, 35, 51). To address whether PI3-K inhibition affects IL-2-driven cell cycle progression, we analyzed the cell cycle distribution in wortmannin-treated CTLL-2 cells. Because PI3-K levels remained fully inhibited 9 h after a single addition of wortmannin (100 nM) but began to recover by 12 h (data not shown), a 12-h time point was used for analysis. Exponentially growing CTLL-2 cells were treated with medium, wortmannin, or the potent inhibitor of T-cell proliferation, rapamycin, for 12 h, and cell cycle distribution was analyzed (Table 1). As has been reported previously (5, 16, 39), rapamycin-treated cells accumulated in the G_0/G_1 phase of the cell cycle, with a corresponding loss of cells in S phase. Wortmannin also increased the number of cells in the G_0/G_1 phase of the cell cycle, with a concurrent reduction of cells in S phase, suggesting that PI3-K activity contributes to IL-2-induced progression through G_1.

TABLE 1. Effect of wortmannin on exponentially growing CTLL-2 cells

Treatment	Cell cycle distribution (% of total)[a]		
	G_0/G_1	S	G_2/M
None	45	48	6
100 nM wortmannin	59	32	9
10 nM rapamycin	69	20	12

[a] Exponentially growing CTLL-2 cells were treated with medium, wortmannin, or rapamycin. Cells were then stained with propidium iodide, and cell cycle parameters were determined by flow cytofluorimetry.

Because wortmannin-treated cells accumulated in the G_0/G_1 phase of the cell cycle, we asked whether PI3-K inhibition affected the IL-2-induced G_1- to S-phase transition. Growth factor-deprived, G_1-synchronized CTLL-2 cells were pretreated with wortmannin or rapamycin, restimulated with the indicated concentrations of IL-2 for 12 h, and incubated with [^{3}H]thymidine for 6 h (Fig. 2). Under these conditions, the cells begin to enter S phase in a relatively synchronous fashion at 11 to 12 h after addition of IL-2 (39). Therefore, during the initial phase of IL-2 stimulation, which is crucial for IL-2-induced S-phase entry (10), PI3-K remained maximally inhibited. These results showed that rapamycin, and to a lesser extent wortmannin, suppressed IL-2-induced DNA synthesis. Additionally, these results suggest that a wortmannin-sensitive PI3-K activity was not essential for IL-2-induced S-phase entry; however, PI3-K was required for optimal G_1- to S-phase progression.

IL-2-induced pp70^{S6K} activation in PI3-K-inhibited CTLL-2 cells. Wortmannin inhibits pp70^{S6K} activation induced by the tyrosine kinase receptors for platelet-derived growth factor (PDGF) and insulin (11, 12). Consequently, we asked whether wortmannin would also inhibit pp70^{S6K} activation mediated by the IL-2R, a member of the cytokine receptor family. Growth factor-deprived CTLL-2 cells were pretreated with medium or the indicated concentrations of wortmannin or rapamycin. The pretreated cells were stimulated with either IL-2 or PMA, and pp70^{S6K} was immunopurified from cleared lysates (Fig. 3). Both IL-2 and PMA elicited pp70^{S6K} activation in untreated cells. The IL-2-induced pp70^{S6K} activation was completely inhibited by wortmannin, whereas the drug only partially inhibited PMA-provoked pp70^{S6K} activation. In agreement with previous reports (13, 27), rapamycin not only decreased the basal level of pp70^{S6K} activity but also completely blocked both

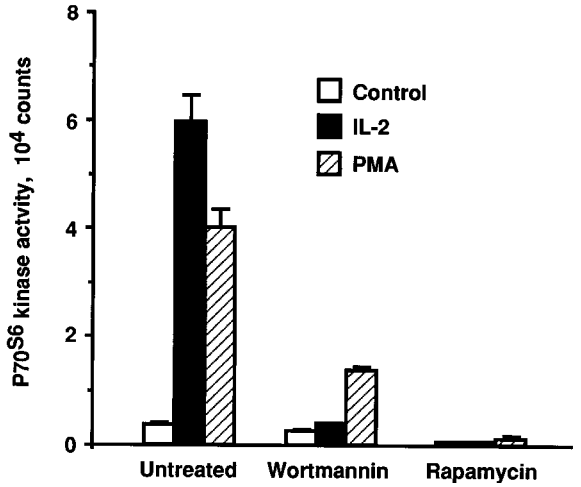

FIG. 3. Activation of pp70^{S6K} in wortmannin- and rapamycin-pretreated CTLL-2 cells. Growth factor-deprived cells were pretreated with medium only or the indicated concentrations of wortmannin or rapamycin. Cells were then restimulated with IL-2 (200 U/ml) or PMA (100 ng/ml) for 20 min, and pp70^{S6K} was immunoprecipitated from cleared detergent lysates. Immunoprecipitates were subjected to kinase reactions and were fractionated by SDS-PAGE (15% polyacrylamide gel). Radiolabeled substrate was quantitated with an Ambis imaging system. Datum points represent counts accumulated in a 1-h acquisition period and are the means of duplicate determinations ± variance.

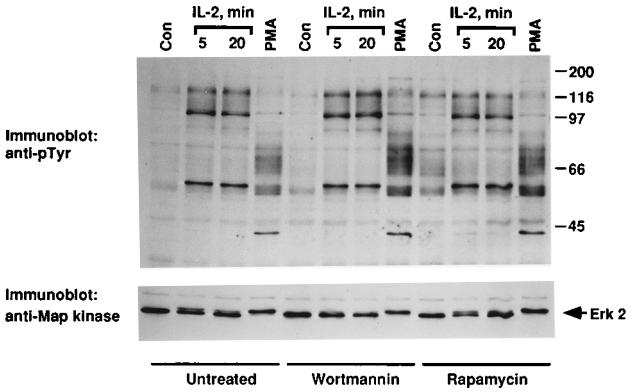

FIG. 4. IL-2- and PMA-induced protein tyrosine phosphorylation. CTLL-2 cells were deprived of growth factors for 5 h; pretreated with medium only (Con), 100 nM wortmannin, or 10 nM rapamycin; and then restimulated either with IL-2 (200 U/ml) for 5 or 20 min or with PMA (100 ng/ml) for 5 min. Detergent-soluble proteins were fractionated by SDS-PAGE (10% polyacrylamide gel) and were transferred to Immobilon-P. The membrane was immunoblotted with an anti-pY monoclonal antibody and then stripped and immunoblotted with an anti-MAP kinase antiserum. Molecular mass markers (in kilodaltons) are indicated for the anti-pY blot. The MAP kinase 42-kDa isoform, ERK2, is indicated in the anti-MAP kinase blot.

the IL-2- and PMA-induced activation of pp70^{S6K}. These results suggest that catalytically active PI3-K is requisite for both IL-2- and PMA-induced pp70^{S6K} activation. However, a portion of the PMA-triggered pp70^{S6K} activating signal was not inhibited by wortmannin.

PI3-K inhibition does not affect IL-2-triggered receptor-proximal activation events. PI3-K has been implicated in the PDGF-induced internalization of the PDGF receptor (PDGF-R [23]). Therefore, we determined whether inhibition of PI3-K catalytic activity would affect ligand-triggered IL-2R internalization. Exponentially growing CTLL-2 cells were stripped of bound IL-2 and pretreated with medium only or 100 nM wortmannin. Radioiodinated IL-2 was prebound to the cells, and IL-2R internalization was quantitated. In contrast to the PDGF-R, ligand-triggered IL-2R internalization was not affected by wortmannin (data not shown), suggesting that this response does not require catalytically active PI3-K.

The earliest detectable event in IL-2-stimulated cells is the triggering of PTK activity. To ascertain whether wortmannin influenced IL-2-stimulated PTK activation, we analyzed IL-2- and PMA-induced protein tyrosine phosphorylation in drug-pretreated CTLL-2 cells. Cells were stimulated with IL-2 or PMA for the indicated times, and cleared lysates were fractionated by SDS-PAGE. Anti-pY immunoblots demonstrated that neither wortmannin nor rapamycin inhibited protein tyrosine phosphorylation induced by IL-2 or PMA (Fig. 4, upper panel), suggesting that PI3-K is not required for PTK activation by either stimulus.

PI3-K is required for maximal IL-2-triggered MAP kinase activation. Studies with other model systems have suggested a role for PI3-K in receptor-induced MAP kinase activation (40, 60, 67). The availability of wortmannin allowed us to use a pharmacological approach to examine the role of PI3-K in IL-2-induced MAP kinase activation. The anti-pY blot shown in Fig. 4 (upper panel) was probed with an anti-MAP kinase antiserum (Fig. 4, lower panel). Phosphorylations that activate MAP kinase also retard the enzyme's electrophoretic mobility during SDS-PAGE. Both IL-2 and PMA induced the appearance of a more slowly migrating form of MAP kinase in both untreated and rapamycin-treated CTLL-2 cells. In contrast, the magnitude of the IL-2- but not the PMA-provoked mobility shift was significantly reduced in wortmannin-pretreated cells. To directly assess wortmannin's effects on IL-2-triggered MAP kinase activation, we assayed MAP kinase catalytic activity in vitro with a peptide substrate. Growth factor-deprived CTLL-2 cells were pretreated with the indicated concentrations of wortmannin (Fig. 5A) and then stimulated with medium or IL-2. MAP kinase was partially purified from cell lysates, and the catalytic activity was quantitated. Pretreatment of CTLL-2 cells with 100 nM wortmannin decreased IL-2-induced MAP kinase activation by greater than 50% (Fig. 5B). Treatment with higher concentrations of wortmannin did not further sup-

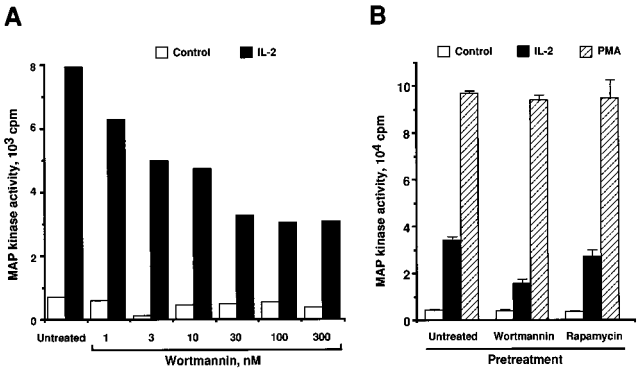

FIG. 5. IL-2- and PMA-induced MAP kinase activation. (A) Growth factor-deprived CTLL-2 cells were pretreated with medium only (untreated) or with the indicated concentrations of wortmannin for 20 min. The cells were restimulated with medium only (control) or 200 U of IL-2 per ml for 5 min. Cell lysates were prepared, MAP kinase was partially purified, and MAP kinase catalytic activity was assayed. (B) Growth factor-deprived CTLL-2 cells were pretreated with nothing (untreated), 100 nM wortmannin, or 10 nM rapamycin, as indicated, for 30 min. Cells were then stimulated with medium only (control), 200 U of IL-2 per ml, or 100 ng of PMA per ml for 5 min. MAP kinase activities were assayed as described for panel A.

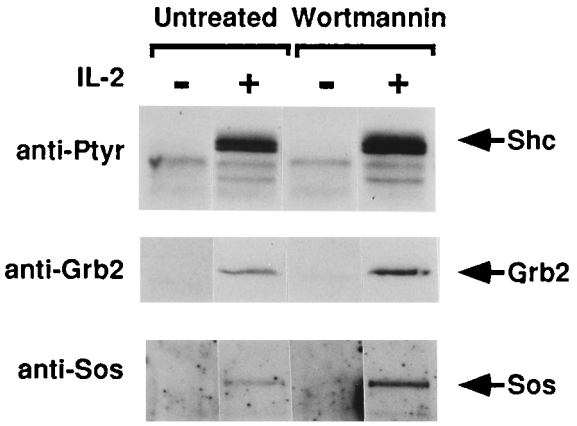

FIG. 6. IL-2-induced Shc tyrosine phosphorylation and Grb2 and Sos association. Growth factor-deprived CTLL-2 cells were pretreated with medium only (untreated) or 100 nM wortmannin for 30 min. Cells were then restimulated with medium only (−) or with 200 U of IL-2 per ml (+) for 5 min. Detergent lysates were immunoprecipitated with anti-Shc antiserum and protein A-Sepharose. Washed immunoprecipitates were fractionated by SDS-PAGE (10% polyacrylamide gel) and transferred to Immobilon-P. The membrane was then sequentially immunoblotted with anti-Grb2, anti-Sos, and anti-pY antibodies, with a stripping step interposed between each round of blotting. The migration position for each protein is indicated by a labeled arrow.

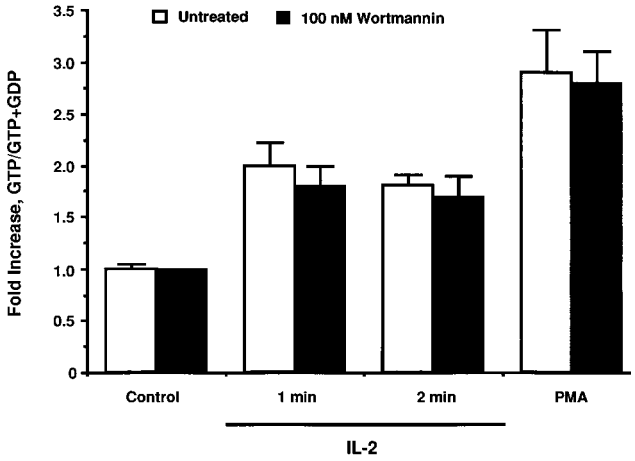

FIG. 7. IL-2- and PMA-provoked Ras activation. Growth factor-deprived, [^{32}P]P_i-labeled CTLL-2 cells were pretreated with medium only (untreated) or 100 nM wortmannin. Cells were then stimulated with medium (control), IL-2 for 1 or 2 min, or PMA for 10 min. Ras was immunoprecipitated from detergent lysates with anti-Ras monoclonal antibody Y13-259. Precipitates were washed, and Ras-bound guanine nucleotides were eluted and were resolved on polyethyleneimine-cellulose thin-layer chromatography plates. Radiolabeled GTP and GDP were quantitated with an Ambis imaging system. Data are expressed as a fold increase in the ratio of radiolabeled GTP to GTP-GDP bound to Ras. Bars are the means ± standard errors of the means of three independent experiments.

press the IL-2-induced MAP kinase activation. The approximate 50% inhibitory concentration for wortmannin in this assay was 3 nM, which correlates closely with that found for PI3-K inhibition (Fig. 1). To demonstrate that MAP kinase inhibition was not due to nonspecific effects of the drug on MAP kinase itself, we utilized the alternative stimulus, PMA (Fig. 5B). IL-2- but not PMA-induced MAP kinase activation was attenuated by pretreatment with 100 nM wortmannin. In addition, rapamycin pretreatment did not affect MAP kinase activation elicited by either stimulus. To demonstrate that the inhibitory effect of wortmannin was not restricted to CTLL-2 cells, we also used a clonal IL-2-dependent human T-cell line (63). In these cells, wortmannin also inhibited IL-2-induced MAP kinase activation to a similar extent (results not shown). Additionally, we have also obtained similar results in anti-MAP kinase immunoprecipitates with myelin basic protein as a substrate. In these studies, IL-2 stimulated a 13.3-fold increase in MAP kinase activity in untreated CTLL-2 cells, whereas in wortmannin-pretreated cells, IL-2 induced a 5.9-fold stimulation of MAP kinase. Furthermore, PMA provoked a 26-fold versus a 29-fold activation of immunopurified MAP kinase in untreated and wortmannin-treated cells, respectively. Taken together, these results implicate a wortmannin-sensitive step in the IL-2-induced activation of MAP kinase in both murine and human T cells.

PI3-K activity is not required for IL-2-induced Ras activation. Because IL-2- but not PMA-provoked MAP kinase activation was sensitive to wortmannin pretreatment, we determined whether the proximal events required for IL-2-induced Ras activation were sensitive to PI3-K inhibition. Like other PTK-dependent growth factors, IL-2 elicits rapid tyrosine phosphorylation of Shc and the attendant association of the Grb2-SOS complex with phosphorylated Shc (8, 50, 70), thereby initiating the activation of Ras. Therefore, we asked whether IL-2-provoked phosphorylation of Shc, and the consequent association of Shc with Grb2 and Sos, was altered by wortmannin pretreatment. Growth factor-deprived CTLL-2 cells were pretreated with medium only or with 100 nM wortmannin, and the cells were restimulated with IL-2 (Fig. 6). Shc was immunoprecipitated from detergent lysates, fractionated by SDS-PAGE, and transferred to Immobilon-P. The membrane was sequentially immunoblotted for Sos, Grb2, and pY. IL-2 induced both the tyrosine phosphorylation of Shc and the association of Shc with the Grb2-Sos complex. These data suggest that wortmannin does not interfere with the coupling of the IL-2R to the Shc-Grb2-SOS complex in T cells.

Subsequent studies were performed to determine whether IL-2-induced Ras activation was sensitive to inhibition by wortmannin. Growth factor-deprived, [^{32}P]P_i-labeled CTLL-2 cells were pretreated with medium, 100 nM wortmannin, or 9 μM herbimycin A and then restimulated with IL-2 or PMA. Ras was immunoprecipitated from the cleared lysates, and the bound nucleotides were eluted and fractionated by thin-layer chromatography (Fig. 7). In the absence of drug, IL-2 triggered a twofold increase in GTP-bound Ras (expressed as a ratio of GTP to GTP plus GDP), and wortmannin had no effect on GTP accumulation. However, herbimycin A-mediated inhibition of PTK activity resulted in almost complete inhibition of Ras activation (data not shown). These results suggest that IL-2-induced Ras activation is not sensitive to PI3-K inhibition by wortmannin.

IL-2-induced activation of MEK requires PI3-K catalytic activity. The serine-threonine kinases Raf and MEK relay the intracellular signal from Ras to MAP kinase. Because IL-2-induced MAP kinase activation, but not Ras activation, was sensitive to wortmannin, we first determined whether IL-2-induced MEK activation required catalytically active PI3-K. Growth factor-deprived CTLL-2 cells were pretreated with medium or 100 nM wortmannin. The cells were then restimulated with IL-2 or PMA, and MEK was immunoprecipitated from the cleared lysates. MEK catalytic activities were determined by incubating the washed immunoprecipitates with [γ-^{32}P]ATP and kinase-dead GST-ERK1 as a MEK substrate. Reaction products were fractionated by SDS-PAGE and transferred to Immobilon-P, and incorporation of radioactivity into kinase-dead GST-ERK1 substrate was quantitated (Fig. 8). In

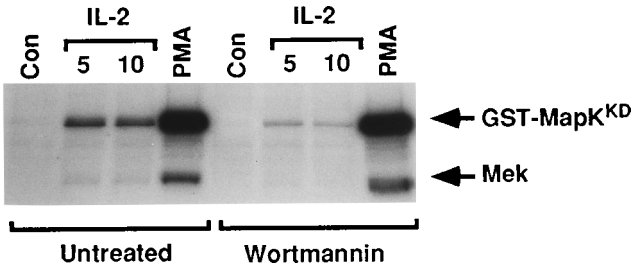

FIG. 8. IL-2-induced MEK activation. Growth factor-deprived CTLL-2 cells were pretreated with medium only or with 100 nM wortmannin for 30 min. Cells were then stimulated with medium (Con), with IL-2 for 5 or 10 min, or with 100 ng of PMA per ml for 5 min. MEK immunoprecipitates were incubated with [γ-^{32}P]ATP and the exogenous substrate, kinase-dead GST-MAP kinase (labeled GST-MapK^{KD}). Reaction products were fractionated by SDS-PAGE, transferred to Immobilon-P, and visualized by autoradiography. Radioactivity in each band was quantitated with an Ambis imaging system. From left to right, 110, 2,400, 2,600, 28,200, 280, 540, 600, and 32,200 counts were accumulated in a 3-h data acquisition period. The experiment was repeated twice with similar results. The migration position of MEK is also indicated.

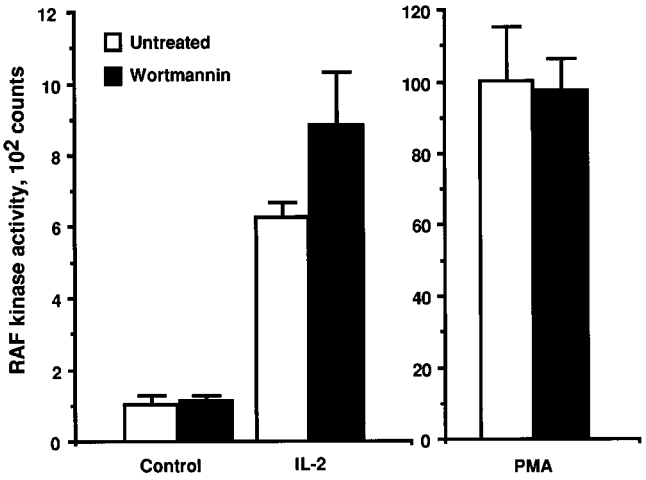

FIG. 9. IL-2-triggered Raf activation. Growth factor-deprived CTLL-2 cells were pretreated with medium or 100 nM wortmannin and were stimulated with PMA or IL-2 for 5 min. Catalytic activity of immunopurified Raf was assessed in duplicate samples by incubation of the washed precipitates with [γ-^{32}P]ATP and kinase-dead GST-MEK as substrate. Kinase reaction products were fractionated by SDS-PAGE, and radioactivity incorporated into the substrate was quantitated with an Ambis imaging system. The data were corrected for nonspecific background radioactivity in nonimmune rabbit anti-mouse immunoglobulin G immunoprecipitates from PMA-stimulated cells. Bars represent means ± variances from duplicate samples of the cumulative counts acquired in a 2-h period.

untreated cells, IL-2 induced a 20-fold increase in the activity of MEK. However, IL-2 triggered only a threefold increase in MEK activity in wortmannin-pretreated cells. As was observed with MAP kinase, wortmannin pretreatment had no effect on PMA-provoked MEK activation. Parallel studies were also performed with the structurally distinct PI3-K inhibitor, LY294002 (61). At a concentration previously reported to inhibit PI3-K activity (11), this drug also suppressed IL-2- but not PMA-induced MEK activation (data not shown). Further studies revealed that addition of 100 nM wortmannin to anti-MEK immunoprecipitates, under conditions that abrogated immunopurified PI3-K activity, did not inhibit basal, IL-2-, or PMA-stimulated MEK catalytic activity (data not shown), demonstrating that wortmannin does not directly inhibit MEK. Taken together, these results suggest that PI3-K catalytic activity is required for full IL-2-induced MEK activation.

PI3-K is not requisite for IL-2-provoked Raf activation. IL-2-triggered MEK, but not Ras activation, was sensitive to PI3-K inhibition. Therefore, we asked whether wortmannin also attenuated the IL-2-induced activation of Raf, the protein kinase that links activated Ras to the downstream effector, MEK. Growth factor-deprived cells were pretreated with medium or 100 nM wortmannin and were restimulated with IL-2 or PMA. The catalytic activity of immunopurified Raf was quantitated with a kinase-dead GST-MEK fusion protein as substrate (Fig. 9). Strikingly, IL-2-induced Raf activation was not inhibited in wortmannin-treated cells. Additionally, pretreatment of CTLL-2 cells with LY294002 also did not inhibit IL-2-induced Raf activation (data not shown). These results suggest that the inhibition of IL-2-induced MEK activation is not the result of a corresponding inhibition of the MEK activator, Raf.

DISCUSSION

IL-2R ligation activates multiple signal-transducing cascades in T cells. These include the constituents of the Ras/MAP kinase pathway, as well as the enzymes pp70^{S6K} and PI3-K, which are elements of less well characterized signaling pathways. In other receptor systems, these signaling components have been segregated into linear transduction cascades, with little evidence of interplay among these pathways. However, recent work has begun to blur these demarcations. The discovery of the potent and specific pharmacological PI3-K inhibitor, wortmannin, allowed us to address the role of PI3-K catalytic activity in both IL-2-dependent DNA synthesis and the integration of IL-2-induced signaling events.

Several approaches have been employed to address the role of PI3-K in PTK-dependent signaling. Using wortmannin (37), LY294002 (11, 12), or PDGF-R mutants that selectively disrupt PI3-K activation (12), these groups demonstrated that PI3-K was essential for pp70^{S6K} activation by PTK-dependent receptors. In agreement with these studies, the present data indicate that IL-2- and, to a lesser extent, PMA-induced pp70^{S6K} activation requires catalytically active PI3-K. In addition to its function upstream of pp70^{S6K}, several studies have implied a role for PI3-K in the activation of elements of the Ras/MAP kinase pathway. PDGF-R add-back mutants, which contain only the PI3-K binding sites, are able to activate Ras (60). In addition, overexpression of full-length p85 suppresses insulin-induced activation of the serum response element, which is a MAP kinase-dependent event (67). Finally, an SH2 domain of p85 specifically inhibits progesterone-induced MAP kinase activation in *Xenopus* oocytes (40). Whether such strategies specifically abrogate only receptor-activated, PI3-K-dependent signaling pathways is unknown. As an alternative approach, we utilized wortmannin as a pharmacological probe to address the role of PI3-K in the IL-2-evoked activation of the Ras/MAP kinase pathway.

Initial studies demonstrated that, although wortmannin pretreatment inhibited IL-2-induced MAP kinase activation, it did not affect Ras activation. Further dissection of the protein kinase cascade that relays the signal from Ras to MAP kinase revealed that IL-2-triggered MEK activation is also sensitive to wortmannin, whereas IL-2-evoked activation of Raf, the canonical upstream activator of MEK, was insensitive to the drug. This result suggests that, in addition to Raf, a previously unrecognized PI3-K-dependent pathway also impinges upon MEK. Examples of other MEK-activating kinases include Mos (48) and the recently described MEK kinases (MEKK [28, 29]). The 72-kDa form of MEKK may link heterotrimeric G protein-initiated signals to MAP kinase activation (29),

whereas the 98-kDa MEKK isoform mediates Ras-dependent MEK activation in epidermal growth factor- and nerve growth factor-stimulated PC12 cells (28). The present results suggest that the IL-2R and possibly other cytokine receptors are coupled to a PI3-K-regulated MEKK in lymphoid cells.

The role of Ras in IL-2-dependent MEK activation is a matter of conjecture. In other cell types, the activation of both Raf (66) and the 98-kDa MEKK (28) is Ras dependent. Additionally, PI3-K also appears to be downstream of Ras (53). Ras may provoke the initial signal for both Raf and PI3-K activation, resulting in the relay of Raf-dependent and PI3-K-dependent activating signals to MEK. The differential inhibitory effects of wortmannin on Raf versus MEK activation suggest that the PI3-K-dependent MEK activation pathway is dominant in IL-2-dependent CTLL-2 cells. Using an alternative approach, Zheng et al. have also proposed that Raf regulates only a small proportion of epidermal growth factor-induced MEK activation (69). If the activation of PI3-K is independent of Ras in T cells, then the present results suggest a novel pathway for PTK-dependent signals to bypass Ras and activate MAP kinase. In such a scenario, MEK would integrate and relay MAP kinase-activating signals emanating from both Ras-dependent and PI3-K-dependent upstream pathways.

Several studies have implicated PI3-K in receptor-regulated proliferation (11, 60). Surprisingly, we found that wortmannin-sensitive PI3-K activity was not essential for IL-2-induced S-phase entry; however, PI3-K was required for optimal IL-2-activated DNA synthesis. This discrepancy may reflect the extent to which different receptors and cell types rely on PI3-K for G_1- to S-phase progression. Alternatively, the discordance may result from the different methods used to inhibit PI3-K. Although wortmannin-mediated PI3-K inhibition partially suppressed IL-2-induced DNA synthesis and caused an accumulation of cells in the G_1 phase of the cell cycle, it is difficult to discern whether the antiproliferative effect of the drug is due to the inhibition of pp70^{S6K}, MAP kinase, or a combination of both actions. In the case of pp70^{S6K}, both rapamycin and wortmannin inhibited IL-2-induced pp70^{S6K} activation, and yet IL-2-evoked T-cell proliferation was much more sensitive to rapamycin than to wortmannin. Therefore, these data suggest that the growth-inhibitory effects of rapamycin were not due solely to the inhibition of IL-2-induced pp70^{S6K} activation.

Activation of MEK and MAP kinase has also been implicated in mitogenic signaling from numerous growth factors. However, the relative importance of the Ras/MAP kinase pathway in IL-2-induced DNA synthesis is a matter of speculation. The T-cell growth factor, IL-4, does not activate Ras (17, 54), and yet IL-4 supports the growth of many T-cell lines. Therefore, although IL-2 activates Ras, this event may not be essential for T-cell proliferation. However, the present data demonstrate that PI3-K inhibition partially blocks both IL-2-induced MAP kinase activation and IL-2-evoked DNA synthesis, suggesting that MAP kinase may be a limiting component in the G_1- to S-phase transition in this system, and that the Ras/Map kinase pathway is essential for IL-2R-induced mitogenesis.

These studies begin to elucidate a role for PI3-K in the downstream signaling events elicited by IL-2R ligation. Although PI3-K, MAP kinase, and pp70^{S6K} are commonly assigned to divergent signal transduction pathways, it is becoming evident that these pathways are interdependent. One point of crosstalk among these pathways may be PI3-K itself. PI3-K is required for IL-2-induced pp70^{S6K} activation and also supplies a requisite signal for activation of MEK, a central component in the Ras/MAP kinase pathway. Future studies will determine whether PI3-K directly modulates pp70^{S6K} and MEK activities or whether other signaling enzymes link PI3-K to these downstream enzymes in IL-2-stimulated T cells.

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