Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7571–7575, August 1992
Immunology

Interleukin 2 stimulation of p70 S6 kinase activity is inhibited by the immunosuppressant rapamycin

(cytokines/growth factor signaling/serine/threonine kinases)

VÍCTOR CALVO*†, CRAIG M. CREWS‡, TERRY A. VIK*‡§¶‖, AND BARBARA E. BIERER*†**††

*Division of Pediatric Oncology, Dana–Farber Cancer Institute, §The Children's Hospital, and Departments of ¶Pediatrics and **Medicine, Harvard Medical School, Boston, MA 02115; ‡Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138; and ††Hematology-Oncology Division, Brigham and Women's Hospital, Boston, MA 02115

Communicated by Raymond L. Erikson, May 8, 1992 (received for review April 1, 1992)

ABSTRACT **Binding of interleukin 2 (IL-2) to its receptor generates intracellular signals, including the activation of tyrosine and serine/threonine kinases. In this study the activation of the serine/threonine-specific ribosomal protein S6 kinases in response to IL-2 was analyzed in the murine T-cell line CTLL-20, a model system of IL-2-dependent proliferation. Two major classes of S6 kinases have been characterized: the 90-kDa (rsk) family and the 70-kDa family. In response to the addition of recombinant IL-2, total S6 kinase activity was increased. This S6 kinase activity could not be immunoprecipitated by an antiserum specific for S6 kinases of the 90-kDa family, exhibited a chromatographic behavior characteristic of 70-kDa S6 kinases, and was recognized by a 70-kDa S6 kinase-specific antiserum. Thus, IL-2 binding to its receptor induces specific activation of the 70-kDa family of S6 kinases. Rapamycin, a macrolide immunosuppressant that inhibits IL-2-dependent proliferation, inhibited IL-2-stimulated 70-kDa S6 kinase activity subsequent to early increases in tyrosine kinase activity. These findings imply that the targets of rapamycin include molecules involved in the activation of 70-kDa S6 kinases. These observations further suggest that S6 kinases of the 70-kDa family participate in signal transmission pathways subsequent to IL-2 binding to its receptor.**

Interleukin-2 (IL-2) is a critical regulator of cell cycle progression in T lymphocytes (1). IL-2 binds to a specific cell surface receptor comprised of at least two distinct subunits, the IL-2Rα and IL-2Rβ chains (2–5). The signal transduction events that culminate in the entry of cells into S phase appear to be dependent only on the IL-2Rβ subunit (6–8). The biochemical mechanisms that couple IL-2 receptor occupancy to subsequent proliferative responses are being actively investigated.

A conserved response of many cell types to mitogenic signals is the phosphorylation of the ribosomal protein S6 on multiple serine residues (9). This phosphorylation increases the efficiency of protein synthesis, which appears to be required in several steps of cell cycle progression (9). IL-2 binding has been reported to stimulate S6 phosphorylation and protein synthesis (10). Recently, two families of S6 kinases have been characterized at the enzymatic and molecular levels: the 90-kDa (rsk) S6 kinase family (11) and the 70-kDa S6 kinase family (12, 13). The activity of both types of kinases is regulated by serine/threonine phosphorylation (9), and both are serine/threonine kinases themselves. We performed experiments to determine which of these kinases were responsible for the IL-2-triggered increase in S6 phosphorylation. Here we show that IL-2 stimulation up-regulates total S6 kinase activity. However, S6 kinases of the 90-kDa family, as detected by a specific antiserum, do not become activated by IL-2 stimulation, and microtubule-associated protein 2 (MAP) kinase, an upstream activator of the 90-kDa S6 kinases, is only minimally activated. In contrast, anion-exchange chromatographic analysis of cell lysates revealed an IL-2-stimulated S6 kinase activity peak characteristic of the 70-kDa S6 kinases. Moreover, immunoblotting with a 70-kDa S6 kinase-specific antiserum demonstrated the presence of 70-kDa S6 kinases in the IL-2-stimulated peak, whereas anti-90-kDa S6 kinase immunoreactive proteins eluted elsewhere. These observations imply that activation of members of the 70-kDa S6 kinase family constitutes a downstream event in IL-2-initiated signal transduction pathways.

The immunosuppressant rapamycin has been shown to inhibit cellular proliferation dependent on IL-2 but not to inhibit IL-2 synthesis (14, 15). FK506 is a structural homolog of rapamycin that inhibits early T-cell activation resulting in the inhibition of lymphokine gene induction, apparently by inhibiting the activity of calcineurin, a serine/threonine phosphatase (16, 17). The addition of rapamycin, but not FK506, to the IL-2-dependent T-cell line resulted in the inhibition of basal and IL-2-stimulated activities of the 70-kDa S6 kinases. In contrast, rapamycin did not inhibit the IL-2-stimulated increase in tyrosine kinase activity as detected by antiphosphotyrosine immunoblotting. This suggests that rapamycin inhibits activation of members of the 70-kDa S6 kinase family (see ref. 29) and, when stimulated by IL-2, such inhibition is subsequent to early increases in tyrosine kinase activity.

MATERIALS AND METHODS

Cell Culture and Stimulation of Cells. The IL-2-dependent murine cell line CTLL-20 (American Type Culture Collection) was cultured in RPMI/10% fetal calf serum (FCS) medium as described (18), containing human recombinant IL-2 (12.5–25 units/ml, kindly donated by Hoffmann–LaRoche). Cells were recovered by centrifugation, washed three times with RPMI 1640 medium, resuspended in RPMI/10% FCS at 1–5 $\times$ 10^6 cells per ml, and incubated for 3 hr at 37°C. Cells were left untreated or treated with 100 nM rapamycin, 100 nM FK506, or equivalent ethanol diluent (final concentration, 0.1%) for the last hour and during the subsequent stimulation period. Aliquots of 4–10 $\times$ 10^6 CTLL-20 cells were either left untreated or incubated in the presence of human recombinant IL-2 (25 units/ml) or phorbol 12-myristate 13-acetate (PMA, 50 ng/ml; Sigma) for the indicated times at 37°C and then divided into two aliquots.

***In Vitro* Kinase Assays.** *In vitro* kinase assays were performed essentially as described (18). Cells (1–2 $\times$ 10^6) were spun after stimulation and lysed in 0.2 ml of 10 mM potassium

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Abbreviations: MAP, microtubule-associated protein 2; rsk, 90-kDa ribosomal S6 kinase; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin 2; TCR, T-cell receptor; FKBP, FK506 binding protein.

††To whom reprint requests should be addressed.
‖Present address: Herman B. Wells Center for Pediatric Research, Riley Hospital for Children, Room 2612, Indianapolis, IN 46202.

phosphate, pH 7.05/1 mM EDTA/0.5% Triton X-100/5 mM EGTA/10 mM $MgCl_2$/50 mM β-glycerophosphate/1 mM sodium vanadate/2 mM dithiothreitol/40 μg of phenylmethylsulfonyl fluoride per ml/10 μg of leupeptin per ml/1 μg of pepstatin per ml. After 30 min on ice, nuclei were pelleted by a 5-min Microfuge centrifugation, and supernatants were used for kinase assays.

For the detection of total S6 kinase activity, direct S6 phosphorylation assays were performed with 5 μl of supernatant in a total volume of 30 μl containing 20 mM Tris·HCl (pH 7.25), 10 mM $MgCl_2$, 10–100 μg of bovine serum albumin per ml, 50 μM ATP with 5 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq), and 40S ribosomal subunits as substrate (0.1–0.25 mg/ml) prepared from *Xenopus laevis* as described (19). For S6 kinase assays specific for 90-kDa S6 kinase, 100 μl of supernatant was incubated with 5 μl of the specific rabbit antiserum 125 (20), and immunocomplexes were adsorbed to *Staphylococcus aureus*, washed as described (21), and subjected to S6 phosphorylation assays as described above.

To assay for MAP kinase activity, 5 μl of supernatant was used in a total reaction volume of 30 μl containing 20 mM Tris·HCl (pH 7.25), 10 mM $MgCl_2$, 100 μM ATP with 5 μCi of [γ-^{32}P]ATP, and ≈1 μg of unactivated *Xenopus* 90-kDa S6 kinase (referred to as rsk) obtained as described (21). In all cases kinase reactions were carried out at 30°C for 15 min and analyzed as described (18).

Antiphosphotyrosine Immunoblotting Analysis. Cells (3–8 × 10^6) were washed, lysed, and centrifuged as described (18). The protein concentration in the supernatants was determined by a colorimetric method (22). Equivalent protein amounts of each supernatant were resolved by reducing 8–10% SDS/PAGE and analyzed by immunoblotting with the antiphosphotyrosine monoclonal antibody 4G10 and anti-mouse IgG alkaline phosphatase-conjugated antibody (Promega) as described (18).

Ion-Exchange Chromatography and Anti-70-kDa/90-kDa S6 Kinase Immunoblotting Analysis. Cells (1.4 × 10^8) were lysed in homogenization buffer (5 mM EGTA/1 mM EDTA/10 mM $MgCl_2$/50 mM β-glycerophosphate/2 mM dithiothreitol/0.2% Triton X-100/10% glycerol/1 mM phenylmethylsulfonyl fluoride/10 μg of leupeptin per ml/10 μg of pepstatin per ml, pH 7.3) and centrifuged at 100,000 × *g* for 30 min at 4°C. Supernatants, normalized for total protein content, were applied to a Mono Q (Pharmacia) anion-exchange chromatography column, and bound proteins were eluted in an 18-ml gradient of 0–500 mM NaCl (23). Aliquots (5 μl) of each 0.75-ml fraction were assayed for S6 kinase activity as described above. Aliquots (16 μl) of each fraction were resolved by reducing 8% SDS/PAGE and analyzed by immunoblotting with the 90-kDa-specific rabbit antiserum 125 as described (21) and with the 70-kDa S6 kinase-specific rabbit antiserum (anti-rat S6 kinase; Upstate Biotechnology, Lake Placid, NY), as recommended by the manufacturer, using an anti-mouse IgG alkaline phosphatase-conjugated antibody.

RESULTS

IL-2 Stimulates S6 Kinases of the 70-kDa Family in CTLL-20 Cells. The IL-2-dependent murine T-cell line CTLL-20 proliferates in response to IL-2 stimulation. CTLL-20 cells were allowed to reach plateau phase of growth, extensively washed, and starved of IL-2 for 3 hr. IL-2 deprivation for this period of time did not affect cell viability or subsequent IL-2-sustained proliferation (data not shown). IL-2-deprived CTLL-20 cells were stimulated with either recombinant IL-2 or PMA and total S6 kinase activity was measured in cell lysates by phosphorylation of 40S ribosomal subunits *in vitro* (Fig. 1*A*). IL-2 binding resulted in a time-dependent stimulation of S6 kinase activity, which was detectable at 5 min after addition of IL-2 and increased cumulatively until at least

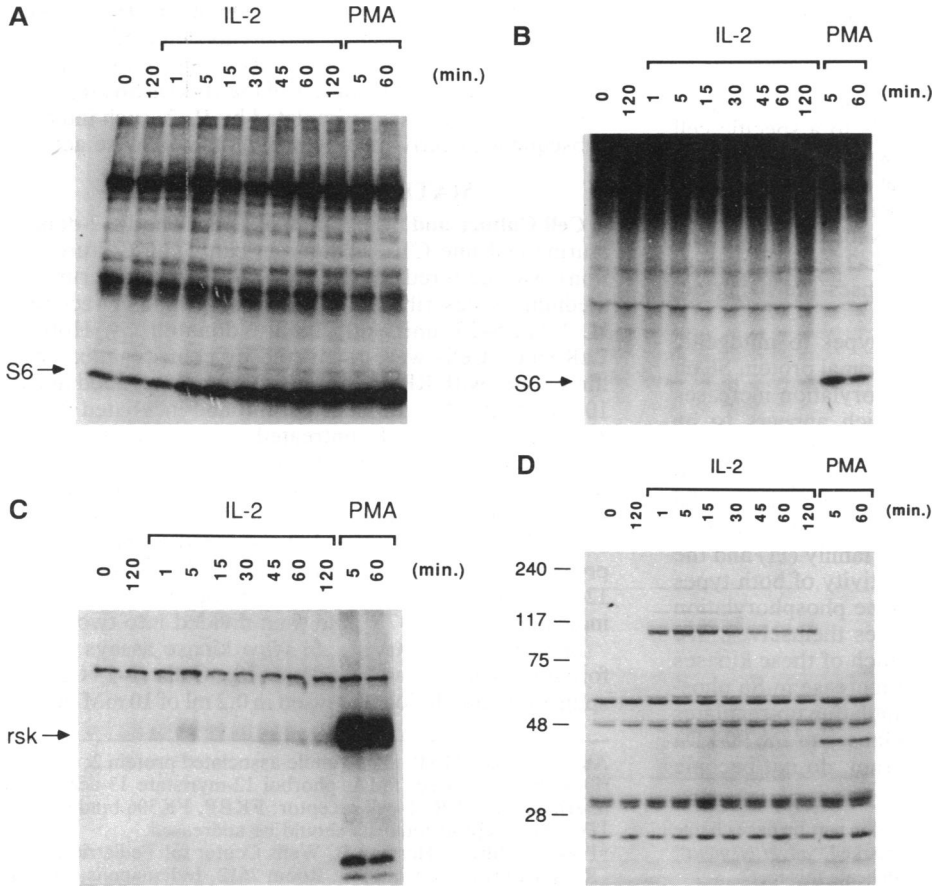

FIG. 1. Effect of IL-2 and PMA on S6 kinase activity, MAP kinase activity, and tyrosine phosphorylation in CTLL-20 cells. Cells were treated with IL-2 or PMA for the indicated times. Lysates were analyzed for total S6 kinase activity (*A*), S6 kinase activity due to 90-kDa S6 kinase (*B*), MAP kinase activity (*C*), or induction of tyrosine phosphorylation (*D*). For the *in vitro* kinase assays (*A–C*), the positions of the corresponding substrates are indicated on the left. For the antiphosphotyrosine immunoblot (*D*), the positions of molecular mass markers (in kDa) are indicated on the left.

120 min after IL-2 addition. Incubation with PMA also resulted in a marked stimulation of S6 kinase activity that increased over time compared to untreated cells.

To analyze the contribution of the 90-kDa S6 kinase to this IL-2-stimulated increase of activity, aliquots of the cell lysates were immunoprecipitated with an antibody specific for 90-kDa S6 kinases (20) and S6 kinase activity was measured in the immunocomplexes (Fig. 1*B*). No time-dependent IL-2-induced increase in S6 phosphorylating activity was detected. However, CTLL-20 cells contained S6 kinase(s) immunoprecipitable by the 90-kDa-specific antiserum, since PMA treatment induced a marked increase in S6 phosphorylating activity measured in the immunocomplex assay following specific immunoprecipitation. Measured PMA-stimulated 90-kDa S6 kinase activity decreased from 5 to 60 min. These observations suggest that IL-2 stimulation does not result in the activation of the 90-kDa S6 kinase, which can nevertheless be stimulated by PMA treatment in these cells. Therefore, the stimulation of S6 kinase activity observed following IL-2 receptor occupancy is most likely the result of activation of members of the 70-kDa S6 kinase family.

MAP kinase has been reported to activate the 90-kDa S6 kinase *in vitro* (24). To determine if the lack of activation of 90-kDa S6 kinase by IL-2 correlated with a lack of activation of MAP kinase, MAP kinase assays were performed using 90-kDa S6 kinase as substrate with lysates from either untreated cells or cells incubated with IL-2 or PMA. MAP kinase activity from lysates of IL-2-stimulated cells was similar to or slightly increased compared to untreated cells (Fig. 1*C* and data not shown). In contrast, PMA-treated cells exhibited a dramatic increase in MAP kinase activity (Fig. 1*C*). These results demonstrate that MAP kinase in CTLL-20 cells, although strongly activated by PMA treatment, is minimally affected by IL-2 stimulation.

IL-2 stimulation induced the appearance of tyrosine-phosphorylated proteins in a time-dependent manner (Fig. 1*D*). A prominent band of ≈100 kDa was observed, as well as a much fainter band of ≈60 kDa. MAP kinase requires phosphorylation on tyrosine and serine/threonine residues to be maximally active (25). Tyrosine-phosphorylated MAP kinase can be detected by antiphosphotyrosine immunoblotting in cells stimulated by various external signals, including T-cell receptor–CD3 (TCR–CD3) stimulation of T cells (18, 26). The presence of tyrosine-phosphorylated 42-kDa protein (the molecular mass of MAP kinase) in IL-2-stimulated cell lysates varied from undetectable to barely detectable (Fig. 1*D* and data not shown). This result correlates with the lack of a prominent stimulation of MAP kinase activity by IL-2. In contrast, incubation with PMA induced a very prominent tyrosine-phosphorylated 42-kDa band (Fig. 1*D*) in all experiments performed.

To obtain direct evidence that the IL-2-stimulated S6 kinase activity corresponded to S6 kinases of the 70-kDa S6 kinase family, cell lysates were fractionated by Mono Q anion-exchange chromatography, which resolves two peaks of S6 kinase activity: 90-kDa S6 kinases elute earlier than 70-kDa S6 kinases in the salt gradient (23). Compared to unstimulated cells, IL-2-stimulated cell lysates contained an increased S6 kinase activity peak that eluted in a position corresponding to 70-kDa S6 kinases (Fig. 2*A*). To verify that 70-kDa and 90-kDa S6 kinases had been effectively separated, we tested for the presence of these kinases in the different fractions by immunoblotting of SDS/PAGE-resolved aliquots with specific antisera. Anti-90-kDa S6 kinase immunoreactive proteins concentrate in fractions 5–11 (Fig. 2*C*), whereas anti-70-kDa S6 kinase immunoreactive proteins concentrate in fractions 14–17 (Fig. 2*B*), which correspond to the peak of IL-2-stimulated S6 kinase activity. Thus, by chromatographic behavior and immunoreactivity,

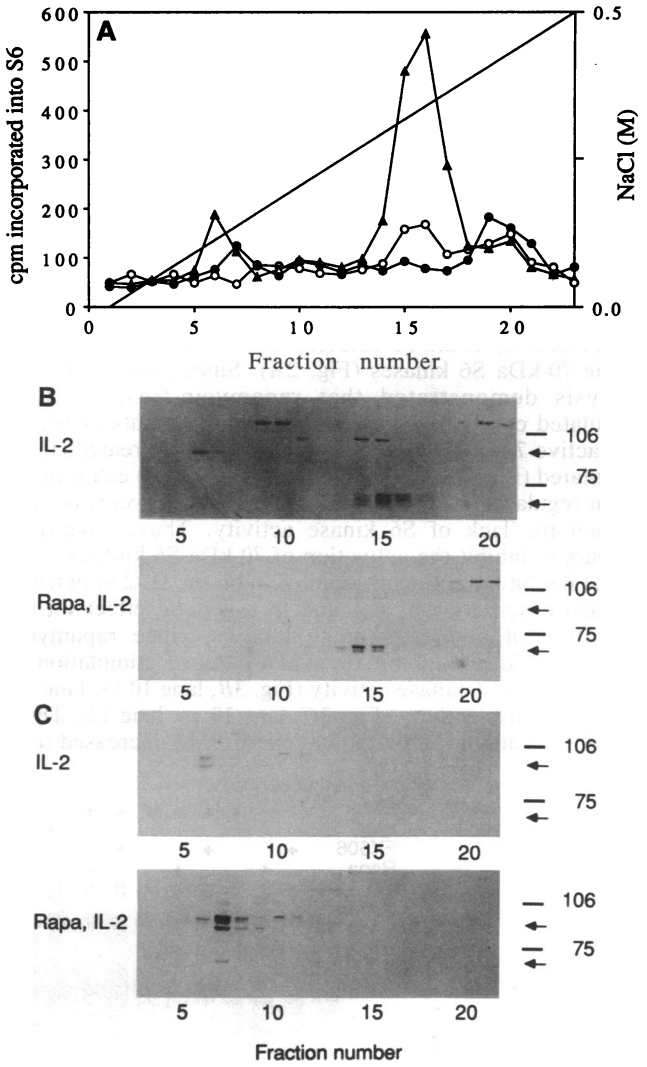

FIG. 2. Chromatographic resolution of 70-kDa and 90-kDa S6 kinases. IL-2-deprived cells were either left untreated or treated with rapamycin (Rapa) for 60 min and either left unstimulated or stimulated with IL-2 for an additional period of 60 min. Cell lysates were applied to a Mono Q anion-exchange column and eluted with a linear gradient of NaCl from 0.0 to 0.5 M; fractions were collected and analyzed. (*A*) S6 kinase activity. ○, Untreated, unstimulated; ▲, untreated, stimulated with IL-2; ●, treated with rapamycin, stimulated with IL-2. (*B*) Immunoblotting with 70-kDa S6 kinase-specific antiserum. (*C*) Immunoblotting with 90-kDa S6 kinase-specific antiserum. Treatments and stimulations are indicated on the left, fraction numbers are indicated at the bottom, and the positions of molecular mass markers (in kDa) are indicated on the right. The positions of 70-kDa and 90-kDa S6 kinases are indicated with arrows on the right. The 90-kDa proteins detected by the anti-70-kDa S6 kinase antiserum (fractions 14 and 15) correspond to the recently described high molecular mass forms of the 70-kDa S6 kinases (23). Note that the protein bands migrating at ≈100–110 kDa that are recognized by the 70-kDa S6 kinase-specific antiserum (fractions 9 and 10 and 19–21) elute in fractions devoid of detectable S6 kinase activity.

IL-2 stimulates an increase in S6 kinase activity of the 70-kDa family.

Inhibition of 70-kDa S6 Kinase Activity by Rapamycin. Rapamycin is a macrolide antibiotic with immunosuppressive activity. Structurally homologous to FK506, rapamycin has been shown to bind to a family of intracellular proteins termed FK506 binding proteins, or FKBPs. Although the FK506/FKBP complex binds to and inhibits the activity of the serine/threonine phosphatase calcineurin (16, 17), the

target of action of rapamycin is unknown. Rapamycin has been shown to inhibit IL-2-dependent T-cell proliferation (14, 15). CTLL-20 cells were treated with rapamycin, FK506, or medium and then stimulated with either IL-2 or PMA. Rapamycin, but not FK506, inhibited not only the time-dependent IL-2-mediated stimulation of total S6 kinase activity (Fig. 3*A*, lanes 6 and 9 vs. lanes 4 and 7) but also basal S6 kinase activity (Fig. 3*A*, lane 3 vs. lane 1). Rapamycin only minimally inhibited the marked stimulation of total S6 kinase activity by PMA (Fig. 3*A*, lane 10 vs. lane 12).

When lysates from rapamycin-treated, IL-2-stimulated cells were fractionated by Mono Q chromatography, no S6 kinase activity could be detected in fractions corresponding to the 70-kDa S6 kinases (Fig. 2*A*). Since immunoblotting analysis demonstrated that rapamycin-treated, IL-2-stimulated cell lysates contained similar amounts of immunoreactive 70-kDa S6 kinases compared to untreated, IL-2-stimulated (Fig. 2*B*) or control (data not shown) cell lysates, down-regulation of 70-kDa S6 kinases by rapamycin does not explain the lack of S6 kinase activity. Thus, rapamycin appears to inhibit the activation of 70-kDa S6 kinases.

The inhibitory action of rapamycin on the IL-2-stimulated S6 kinase activity was not due to a general effect on the activation of serine/threonine kinases, since rapamycin treatment did not inhibit the PMA-induced stimulation of either 90-kDa S6 kinase activity (Fig. 3*B*, lane 10 vs. lane 12) or MAP kinase activity (Fig. 3*C*, lane 10 vs. lane 12). Thus, the modest inhibition by rapamycin of PMA-increased total S6 kinase activity probably reflects the inhibition of the 70-kDa S6 kinase component of the PMA-stimulated S6 kinase activity. Rapamycin did not inhibit the IL-2-induced tyrosine phosphorylation of a number of substrates (e.g., ≈100-kDa band, Fig. 3*D*, lanes 6 and 9 vs. lanes 4 and 7). The target of action of rapamycin thus appears to lie between the early activation of tyrosine kinases and the activation of members of the 70-kDa S6 kinase family, which require serine/threonine phosphorylation for their activation.

DISCUSSION

The observations presented in this report suggest that IL-2-dependent proliferation of CTLL-20 cells is dependent on the activity of the 70-kDa S6 kinases. IL-2 treatment activated an S6 kinase activity in a time-dependent manner. This S6 kinase activity was not immunoprecipitated by an antiserum that recognizes S6 kinases of the 90-kDa (rsk) family (20), exhibited a chromatographic behavior characteristic of 70-kDa S6 kinases, and coeluted with proteins recognized with a 70-kDa S6 kinase-specific antiserum, demonstrating that this activity corresponded to S6 kinases of the 70-kDa family. MAP kinase has been shown to activate 90-kDa S6 kinases (25) and was minimally affected by IL-2 incubation of CTLL-20 cells. MAP kinase and 90-kDa S6 kinases are nevertheless susceptible to activation in these cells, since PMA strongly induced tyrosine phosphorylation of MAP kinase, MAP kinase activation, and 90-kDa S6 kinase acti-

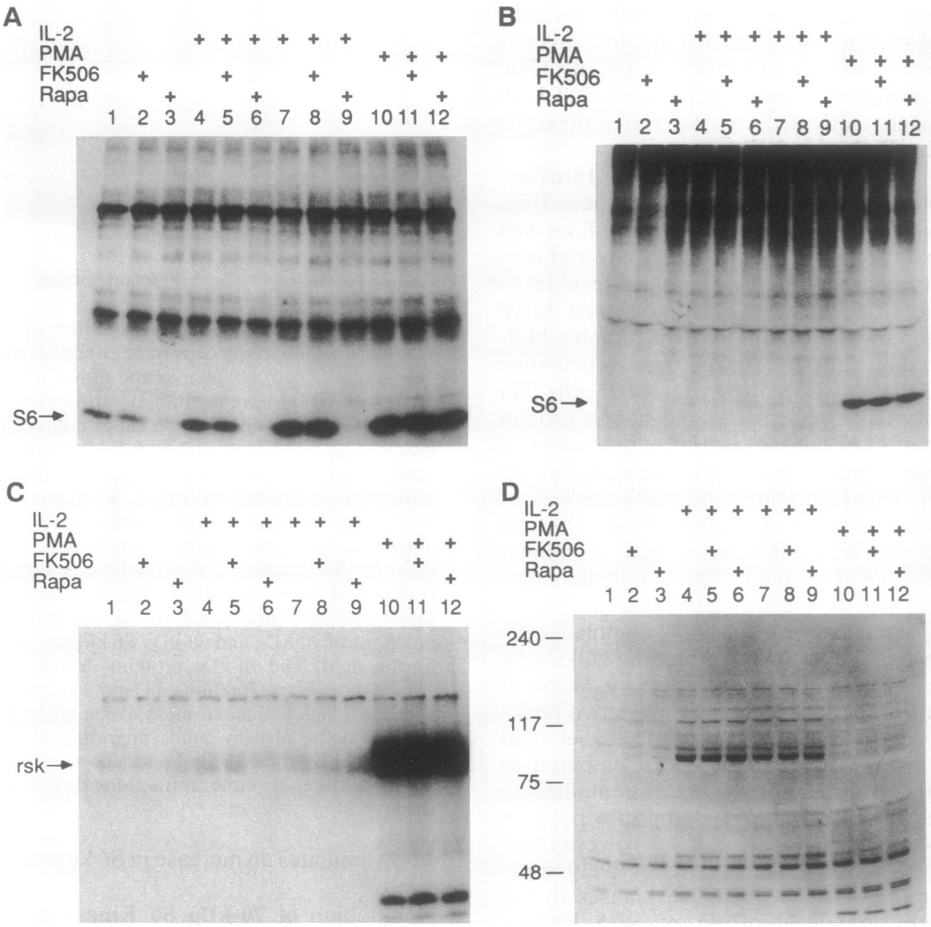

FIG. 3. Effect of rapamycin (Rapa) and FK506 on S6 kinase activity, MAP kinase activity, and tyrosine phosphorylation in CTLL-20 cells. Cells were preincubated with rapamycin or FK506 as indicated and either left untreated for 5 min (lanes 1–3), treated with IL-2 for 5 min (lanes 4–6) or 60 min (lanes 7–9), or treated with PMA for 5 min (lanes 10–12). Lysates were analyzed for total S6 kinase activity (*A*), S6 kinase activity due to 90-kDa S6 kinase (*B*), MAP kinase activity (*C*), or induction of tyrosine phosphorylation (*D*). For the *in vitro* kinase assays (*A–C*), the positions of the corresponding substrates are indicated on the left. For the antiphosphotyrosine immunoblot (*D*), the positions of molecular mass markers (in kDa) are indicated on the left.

vation. These results imply that one of the downstream events triggered by IL-2 binding to its cell surface receptor is the activation of 70-kDa S6 kinases.

The 70-kDa and the 90-kDa S6 kinases have been shown to become activated in response to mitogenic signals in a number of different cell types. We have previously shown that stimulation of the TCR–CD3 complex on the surface of the Jurkat T-cell line induces the activation of 90-kDa, but not 70-kDa, S6 kinases (18). In contrast, the 70-kDa S6 kinases appear to be selectively activated by signals from the IL-2 receptor. Thus, the 90-kDa S6 kinases appear to play a role in early TCR–CD3-triggered signaling events that commit T cells to proliferation, whereas 70-kDa S6 kinases are involved in continued proliferation of T cells in response to IL-2.

The inhibitory action of rapamycin is specific for the 70-kDa S6 kinases, since the 90-kDa S6 kinases, MAP kinase, and the activated tyrosine kinases detected by immunoblotting are unaffected by this drug. Similarly, early TCR–CD3-triggered events resulting in IL-2 secretion are not blocked by rapamycin (14, 15). Rapamycin inhibition of IL-2-dependent proliferation correlated with its inhibition of 70-kDa S6 kinase activity. This correlation has also been observed in nonlymphoid cells: rapamycin specifically inhibited insulin-stimulated proliferation and 70-kDa S6 kinase but not 90-kDa kinase activity in H4 hepatoma cells (29). It should be noted, however, that although rapamycin eradicated basal and stimulated 70-kDa S6 kinase activity, only partial (40–60%) inhibition of proliferation is observed at 24 hr after drug treatment. This suggests that although 70-kDa S6 kinase activity correlated with proliferation, other pathways can be recruited to induce or sustain lymphokine-dependent proliferation. Alternatively, in the absence of S6 phosphorylation by p70 S6 kinase, the rate of proliferation may be decreased. Taken together, these observations suggest that mitogen-stimulated p70 S6 kinase activity is an important component of growth regulation.

Rapamycin binds to a family of intracellular receptors (termed FKBPs), many of which share the capacity to catalyze the cis–trans interconversion of peptidyl-prolyl bonds (rotamase activity) (27). Though drug inhibition of FKBP rotamase activity does not appear to correlate with biological function (28), the formation of a rapamycin–FKBP complex appears to be required for the inhibition of lymphokine-driven proliferation (15, 28). This rapamycin–FKBP complex could interact with any of the upstream activators regulating the activity of the 70-kDa S6 kinases. Since 70-kDa S6 kinase activity is dependent on serine/threonine phosphorylation, the complex of rapamycin bound to FKBP may inhibit the activity of a putative 70-kDa S6 kinase kinase. Alternatively, the rapamycin–FKBP complex may activate a 70-kDa S6 kinase phosphatase that down-regulates p70 S6 kinase activity. In this regard, it is noteworthy that the complex of FK506, a structural homolog of rapamycin, bound to an FKBP (FKBP12) inhibits the activity of calcineurin, a serine/threonine, Ca^{2+}/calmodulin-dependent phosphatase (16, 17). Whether the rapamycin–FKBP complex modulates the activity of a 70-kDa S6 kinase phosphatase, a 70-kDa S6 kinase kinase, or the 70-kDa S6 kinase itself remains to be determined.

We thank T. Roberts and B. Druker for 4G10, D. A. Fruman and W. C. Hahn for review of this manuscript, and S. J. Burakoff for support and review of this manuscript. This work was supported by a postdoctoral fellowship of the Spanish Ministry for Education and Science (V.C.) and by grants from the National Institutes of Health (T.A.V., B.E.B.) and the American Heart Association (B.E.B.).

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