THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 30, Issue of July 25, pp. 28130–28138, 2003
© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Mechanism of Ribosomal p70S6 Kinase Activation by Granulocyte Macrophage Colony-stimulating Factor in Neutrophils

COOPERATION OF A MEK-RELATED, THR[421]/SER[424] KINASE AND A RAPAMYCIN-SENSITIVE, mTOR-RELATED THR[389] KINASE*

Received for publication, January 13, 2003, and in revised form, April 28, 2003
Published, JBC Papers in Press, May 11, 2003, DOI 10.1074/jbc.M300376200

Jason A. Lehman‡, Victor Calvo§, and Julian Gomez-Cambronero‡¶

From the ‡Department of Physiology and Biophysics, Wright State University School of Medicine, Dayton, Ohio 45435 and the §Instituto de Investigaciones Biomedicas, Consejo Superior de Investigaciones Cientificas, Facultad Medicina Universidad Autonoma de Madrid, Arturo Duperier 4, 28029 Madrid, Spain

We report here for the first time the detection of the ribosomal p70S6 kinase (p70S6K) in a hematopoietic cell, the neutrophil, and the stimulation of its enzymatic activity by granulocyte macrophage colony-stimulating factor (GM-CSF). GM-CSF modified the V_{max} of the enzyme (from 7.2 to 20.5 pmol/min/mg) and induced a time- and dose-dependent phosphorylation on p70S6K residues Thr[389] and Thr[421]/Ser[424]. The immunosuppressant macrolide rapamycin caused either a decrease in intensity of phospho-Thr[389] bands in Western blots, or as a downshift in the relative mobility of phospho-Thr[421]/Ser[424] bands (consistent with the loss of phosphate), but not both simultaneously. The immunosuppressant FK506 failed to inhibit p70S6K activation, but was able to rescue the rapamycin-induced downshift, pointing to a role for the mammalian target of rapamycin (mTOR) kinase. Rapamycin also caused an inhibition (IC_{50} 0.2 nM) of the *in vitro* enzymatic activity of p70S6K. However, the inhibition of activity was not complete, but only a 40–50%, indicating that neutrophil p70S6K activity has a rapamycin-resistant component. This component was totally inhibited by pre-incubating the cells with the mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor PD-98059 prior to treatment with rapamycin. This indicated that a kinase from the MEK/MAPK pathway also plays a role in p70S6K activation. Thus, GM-CSF causes the dual activation of a rapamycin-resistant, MAPK-related kinase, that targets Thr[421]/Ser[424] S6K phosphorylation, and a rapamycin-sensitive, mTOR-related kinase, that targets Thr[389], both of which are needed in cooperation to achieve full activation of neutrophil p70S6K.

p70S6K[1] catalyzes the phosphorylation of the S6 protein (1–3), a component of the 40 S subunit of eukaryotic ribosomes. It plays a role in protein synthesis (4) and in cell growth control during G_1 phase, since p70S6K-deficient *Drosophila* and p70S6K knockout mice show significantly reduced body sizes (5–6). Two main S6K isoforms (S6K1 and S6K2) are encoded by different genes (7–11). Ribosomal S6K1 has multiple phosphorylation sites, the most critical residues being Thr[229], Thr[389], and Ser[404] (12, 13). In addition, a "module IV" containing Ser[411], Ser[418], Thr[421], and Ser[424] are phosphorylated in tandem prior to full activation of the enzyme (3). Phosphorylation of Thr[412] and Ser[371] plays a central role in the regulation of p70S6K activity *in vivo* (13, 14).

In the living cell, ribosomal p70S6K is activated through a complex network of signaling molecules (15, 16). The generation of 3-phosphoinositide lipid products by PI3K is required for the phosphorylation of two activating sites in p70S6K: Thr[229] and Thr[389] (17, 18). Thr[229] is phosphorylated by PDK1 (19, 20) and Thr[389] can be phosphorylated by PDK1 (18) but also by several other kinases: NEK6/7 (21), mTOR (also known as FRAP) (12, 22), Akt, and PKCζ (23). The formation of a ternary complex between the regulatory subunit of PI3K (p85), mTOR, and p70S6K is necessary for the activation of the latter (24).

The cytokines IL-3, EPO, and IL-2 induce activation of p70S6K (25–27), as well as cellular proliferation. Both responses are inhibited with rapamycin, an immunosuppressant drug that complexes with FKBP and binds to mTOR, resulting in the dephosphorylation of p70S6K (3, 28). Growth factor-induced activation of p70S6K and G_1 phase cell cycle progression are also blocked by rapamycin or by p70S6K-specific antibodies (25, 26, 29, 30). The structural analog of rapamycin, FK506, competitively binds to FKBP and reverses the inhibition of mTOR by the former (31).

Polymorphonuclear leukocytes (neutrophils) are recruited to sites of inflammation responding to chemoattractants that are secreted by several tissue cells in response to a physical or chemical insult, or by certain bacterial products in the case of a localized infection. The cytokine GM-CSF elicits the two components of cell migration in neutrophils: chemotaxis and chemokinesis.[2] GM-CSF has a number of other functions on neutrophils and their bone marrow precursors, that involve the activation of two major signaling cascades: the JAK/STAT and Ras/MAPK pathways (reviewed in Ref. 33). GM-CSF-induced translocation of p42^{mapk} (ERK2) to the cell nucleus and concomitant phosphorylation of the ribosomal kinase p90^{rsk} is

* This study has been supported by Grant HL056653 from the National Institutes of Health and 0250417N from the American Heart Association (to J. G.-C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Physiology & Biophysics, Wright State University School of Medicine, 3640 Colonel Glenn Highway, Dayton, OH 45435. E-mail: julian.cambronero@wright.edu.

[1] The abbreviations used are: p70S6K, ribosomal p70-S6 kinase; GM-CSF, granulocyte macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; EPO, erythropoietin; IL-8, interleukin-8; PI3K, phosphatidylinositol 3-kinase; PDK1, 3-phosphoinositide-dependent protein kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MEKi, MEK inhibitor PD-98059; mTOR, mammalian target of rapamycin; FKBP, FK506-binding protein; FRAP, FKBP12-rapamycin-associated protein; PP2B, protein phosphatase 2B.

[2] J. Gomez Cambronero, J. Horn, M. A. Baumann, and C. C. Paul, submitted manuscript.

This paper is available on line at http://www.jbc.org

central in mitogenic events. Although it has been shown that G-CSF, a human hematopoietic factor related to GM-CSF, activates PI-3K/Akt(PKB) and promotes cell survival (34), little information exists regarding activation of other members of this cell signaling cascade, particularly p70S6K or whether GM-CSF will mediate its physiological effects (notably cell migration) through mTOR-S6K.

We have previously demonstrated that MAPK activation in response to GM-CSF is up-regulated in mature cells such as the neutrophil and plays a role in chemotaxis (35), and that a molecular connection between the MAPK and the p70S6K pathways exists (36). Here we report that p70S6K is present in neutrophils, that GM-CSF causes an increase in phosphorylation of Thr^{389} and Thr^{421}/Ser^{424} concomitantly to an increase in its enzymatic activity. We also show for the first time that the mechanism by which GM-CSF activates ribosomal S6K is through a combination of activation of two signaling pathways: mTOR and MAPK.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—GM-CSF was from Sandoz (East Hanover, NJ); G-CSF was from Amgen (Thousand Oaks, CA); IL-8 was from R&D Systems (Minneapolis, MN); fMet-Leu-Phe, PKA inhibitor, calphostin-C, PMA, anti-rabbit IgG (agarose beads), the cAMP-dependent kinase inhibitor TTYADFIASGRTGRRNAIHD, anti-p70S6 kinase polyclonal antibody used for immunoblotting, and phalloidin-FITC conjugate conjugate from *Amanita phalloides*, were from Sigma; FACS FLOW buffer was from Fisher (Hanover Park, IL); electrophoresis chemicals were from Bio-Rad Laboratories (Richmond, CA); [γ-^{32}P]ATP (30 Ci/mmol) was from Amersham Biosciences; PD-98059 was from BioMol (Plymouth Meeting, PA); rapamycin, FK506 and alkaline phosphatase (purified from calf intestine) were from Calbiochem (La Jolla, CA); ion-exchange chromatography cellulose phosphate paper was from Whatman (Hillsboro, OR); anti-p70S6 kinase (C-18) polyclonal antibody used for immunoprecipitation was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-phospho(Thr^{389})-p70S6 kinase and anti-phospho(Thr^{421}/Ser^{424})-p70S6 kinase antibodies (polyclonal) were from Cell Signaling (Beverly, MA); p70S6 kinase peptide substrate KKRNRTLTK and 3T3 fibroblasts cell lysates were from Upstate Biotechnology Inc. (Lake Placid, NY).

Cells—Peripheral blood neutrophils were isolated based on a protocol described by English and Andersen (37). Between 50–55 ml of blood were collected from the antecubital vein of healthy individuals (who signed an Institutional Review Board-approved consent form) using sodium citrate as anticoagulant. Blood was mixed with 15 ml of 6% dextran, allowed to settle, and the plasma and buffy coat were removed and spun down at $800 \times g$ for 5 min. The pellet was resuspended in 35 ml of saline and centrifuged again for 15 min at 10 °C in a Ficoll-Histopaque discontinuous gradient. Neutrophils were recovered and contaminating erythrocytes were lysed by hypotonic shock. Cells were washed and the purified neutrophil pellet was resuspended in Hanks Balanced Salt Solution (HBSS). Our experience has indicated that using this protocol, neutrophil aggregation (*i.e.* the hallmark for neutrophil activation) does not occur. Viability is usually $>98 \pm 2\%$ as per trypan blue exclusion. Cells were resuspended at the concentration of 5×10^6 cells/ml in fresh Hanks Balanced Salt Solution (HBSS) or in RPMI at 2×10^6 cells/ml at the time of the experiment, and used within 2–3 h after isolation.

Immunoprecipitation and Western Blotting Analyses—The procedure was based on our previous report (38) with some modifications, as follows. Neutrophils were resuspended in RPMI 1640 at 3×10^6 cells/ml density and were pretreated with rapamycin, FK506, or MEKi where appropriate and then stimulated with GM-CSF at 37 °C. Aliquots (1 ml) were taken, spun down ($14{,}000 \times g$, 15 s) and pellets were resuspended in 0.2 ml of boiling SDS solution (1% SDS in 10 mM Tris-HCl, pH 7.4). Samples were boiled in a heat block for 10 min with frequent vortexing to achieved complete dissolution, taken to an ice bucket and mixed with 0.3 ml cold ddH_2O and 0.4 ml of cold, Triton X-100-based, lysis buffer (12 mM Tris-HCl, pH 7.2, 0.75 mM NaCl, 100 μM sodium orthovanadate, 10 mM phenylmethylsulfonyl fluoride, 0.2 mM β-glycerophosphate, 5 μg/ml each of aprotinin, pepstatin A, and leupeptin, and 0.12% Triton X-100). The resulting 1 ml of total cell lysates were spun down ($14{,}000 \times g$, 1 min, 4 °C) to remove any insoluble material and then used for immunoprecipitation. For this, the primary antibody (anti-p70S6K) was previously mixed at a final concentration of 2 μg/ml with anti-rabbit (IgG, whole molecule) antibody conjugated to agarose beads in lysis buffer for 4 h at 4 °C. The beads were then thoroughly washed and mixed with total cell lysates prepared as indicated above at a ratio agarose beads/cell lysates 1:8 (v/v). After a 2-h incubation period at 4 °C, immune complexes were recovered by centrifugation ($7{,}000 \times g$, 1 min, 4 °C). Pellets were washed twice with lysis buffer, twice with buffer A (100 mM Tris-HCl, pH 7.4, 400 mM LiCl) and twice with buffer B (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). Immune complex beads were resuspended in a final volume of 60 μl with lysis buffer and mixed with 2×-SDS sample buffer (1:1 v/v) for subsequent protein gel electrophoresis/immunoblotting. Resulting gels were transferred onto polyvinylidene difluoride membranes and used for immunoblotting. In several experiments, parallel blots were probed with the same antibody used for immunoprecipitation, to confirm that protein loading was similar (kept at $<5\%$ by measuring protein in samples by Bradford assay before loading) and that the small, unavoidable, differences in protein per lane can not account for differences in phosphorylation seen with the anti-phosphoantibodies.

Immunocomplex p70S6 Kinase Assay—Ribosomal p70S6K enzymatic activity was quantified by using an immunocomplex kinase assay as reported previously (35, 36, 38) tailored to measure this particular kinase activity in human neutrophils. Neutrophils were resuspended in RPMI 1640 at 3×10^6 cells/ml density and were pretreated with rapamycin, FK506, or MEKi where appropriate and then stimulated with GM-CSF at 37 °C. Cells were spun down ($14{,}000 \times g$, 15 s) and pellets were resuspended in 0.3 ml of ice-cold, TRIS-based, lysis buffer (see above for composition) and incubated on ice for 15 min with occasional vortexing. Lysates were obtained after centrifugation ($7{,}000 \times g$, 1 min, 4 °C) in supernatants were mixed with the antibody conjugated to agarose beads as indicated above. Immune complex beads were resuspended in a final volume of 40 μl with of ice-cold lysis buffer (diluted 1:10) and used in an *in vitro* kinase assay. For this, the phosphoacceptor peptide substrate for this assay was 75 μM of the S6 kinase substrate peptide KKRNRTLTK in freshly prepared kinase buffer (13.4 mM HEPES, pH 7.3, 25 mM $MgCl_2$, 30 μM Na_2VO_3, 5 mM *p*-nitrophenyl phosphate, 2 mM EGTA, 2 μM cAMP-dependent kinase inhibitor TTYADFIASGRTGRRNAIHD, 0.420 μCi [γ-^{32}P]ATP (7 nM), and 68 μM unlabeled ATP). 1 μg of cAMP-dependent kinase inhibitor inhibits 2,000–6,000 phosphorylating units of PKA (equivalent to the transference of 2–6 nmol of phosphate from ATP). To initiate the phosphotransferase reaction, aliquots (20 μl) of kinase buffer containing the appropriate substrates were mixed 1:3 (v/v) with the cell lysates or immunocomplex beads. The reaction was carried out at 37 °C for 20 min in a rotator and terminated by blotting 40 μl of the reaction mixture onto P81 ion exchange chromatography cellulose phosphate papers. Filter squares were washed, dried, and counted for radioactivity. Controls were run in parallel with no S6 kinase substrate peptide. Counts were subtracted from samples. In some experiments, ribosomes were used as the natural p70S6K substrate following the *in vitro* kinase assay just indicated. Protein S6 is part of a multiprotein-rRNA complex that is multiphosphorylated on Ser residues *in vitro* in response to mitogenic stimulation. 40 S ribosomal subunits were prepared from *Xenopus laevis* at the concentration of 0.1–0.25 mg/ml following the procedure described in Ref. 11.

Alkaline Phosphatase Treatment—SDS-boiled samples as indicated in the immunoprecipitation and Western blotting analyses section above were diluted with 0.3 ml of cold H_2O and 0.4 ml cold, Triton X-100-based, lysis buffer. The alkaline phosphatase enzyme (calf intestine-purified) was added in a 160 μl of total volume of freshly prepared, cold, alkaline phosphatase buffer (25 mM Tris-HCl, pH 7.6, 1mM $MgCl_2$, and 0.1 mM $ZnCl_2$). To initiate the phosphate removal reaction, 48 μl of 3 M Tris-base, pH 12.5 were added to each sample to achieve a favorable reaction pH of 10.0 ± 0.5. Samples were lightly vortexed and incubated at 37 °C for 45 min with slight agitation. The reaction was stopped by placing the samples on an ice bucket and adding to each reaction tube a small volume of 10 M Tris-HCl, pH 3.0 in order to bring the reaction pH to 7.0. Samples were used immediately for immunoprecipitation using anti-p70S6K antibodies, as indicated above.

F-actin Measurement by Flow Cytometry—Neutrophils were stained with phalloidin-FITC as described (39) with some modifications. Briefly, F-actin polymerization was initiated *in vivo* by the addition of GM-CSF to a neutrophil cell suspension (5×10^6 cells/ml) for 5 min at 37 °C. After this, 0.2-ml aliquots were taken and mixed with 1 ml of pre-chilled fixing solution (two parts of double-concentrated phosphate buffer, pH 7.4, one part of 20% formaldehyde and one part of 75% glycerol in water). Samples were stored at −80 °C until ready for flow cytometry. At that time, samples were thawed and spun down for 5 min at $600 \times g$ in a refrigerated Eppendorf microcentrifuge. Pellets were

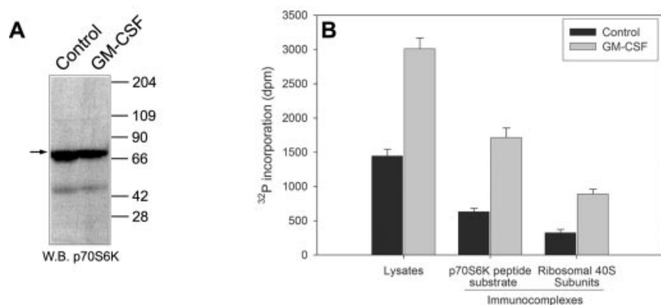

FIG. 1. **p70S6K protein and its activity in human neutrophils.** *A*, presence of p70S6K protein in human neutrophil cells by Western blotting. Neutrophils were resuspended at the density of 1×10^7 cells/ml and incubated with either buffer (*Control*) or with 1 nM GM-CSF (*GM-CSF*) for 3 min. Cell lysates were obtained and analyzed by SDS/PAGE gel electrophoresis followed by immunoblotting. Western blots were probed with anti-p70S6K antibodies. Molecular mass (in kDa) protein standards are marked on the *right*. *B*, detection of p70S6K enzymatic activity in human neutrophil cells. Cells were incubated in the absence (*black bars*) or in the presence of GM-CSF (*gray bars*). Cells lysates were divided into three sets. The first set (*lysates*) was used as such to measure p70S6K activity against 75 μM of the S6 kinase substrate peptide KKRNRTLTK. The second set was subjected to immunoprecipitation (*immunocomplexes*) with anti-p70S6K antibodies, followed by an *in vitro* kinase activity assay in the presence of [γ-^{32}P]ATP and either 75 μM of KKRNRTLTK (*p70S6K peptide substrate*) or 40 S ribosomal subunits from *Xenopus laevis* at the concentration of 0.1–0.25 mg/ml (*ribosomal 40S subunits*). *Panel A* shows a representative experiment among four. Results for *B* are the mean ± S.E. of three independent experiments performed in duplicate.

resuspended in freshly prepared F-actin staining solution (35 μl of a 3.3 mg/ml methanol FITC-phalloidin stock plus 315 μl of H_2O, and stained in the dark for 30 min at room temperature. Samples were centrifuged as above, and pellets were resuspended in 1 ml of FACS FLOW. They were then analyzed by flow cytometry on a FACSCAN Becton & Dickinson flow cytometer at 488 nm excitation wavelength. Data was analyzed using *Cell Quest* software and expressed as fluorescence intensity.

RESULTS

Human Neutrophils Express p70S6K and GM-CSF Increases Its Activity and Phosphorylation Status—We demonstrate here that neutrophils express this protein in sufficient amounts to be clearly detected by immunoblotting using antibodies directed against the ribosomal p70S6K (Fig. 1*A*). A major band shows the predicted molecular weight for the mammalian S6K1 isoform. We were also able to detect its enzymatic activity *in vitro* in whole cell lysates and in anti-p70S6K immunoprecipitates. The detection of p70S6K activity is presented in Fig. 1*B*, with GM-CSF causing a significant and reproducible increase of total S6K activity. Accurate confirmation of ribosomal p70S6K activity is given by the fact that ^{32}P incorporation into the substrate peptide KKRNRTLTK and ribosomal 40 S subunits (containing the natural substrate protein S6), was observed after anti-p70S6K immunoprecipitation. GM-CSF increased basal activity by ~3-fold in immunocomplexes. The complete biochemical characterization of p70S6K in neutrophils is presented in Fig. 2, *A–D*. A ~3-fold activation due to GM-CSF is consistently seen when activity is expressed as a function of time, protein concentration and substrate concentration (Fig. 2, *A–C*). Moreover, there is a considerable change in V_{max} (from 7.2 to 20.5 pmol/min/mg protein) and also in the K_m (from 26.1 to 15.4 μM) by GM-CSF stimulation (Fig. 2*D*) that makes GM-CSF a *bona fide* p70S6K physiological stimulator in neutrophils.

After protein, activity and kinetic measurements, we next investigated whether GM-CSF was able to induce the phosphorylation of p70S6K in human neutrophils. As seen in Fig. 3*A*, GM-CSF induces a rapid (3 min) phosphorylation of p70S6K on Thr^{389}, which is one of the residues critical for S6K activation. The migration of the phosphorylated band in SDS gels very well coincides with that of the 3T3 fibroblast controls. Also, GM-CSF induces phosphorylation of p70S6K on two other residues, Thr^{421}/Ser^{424} (Fig. 3*B*), that are also key for conferring enzyme activity. The effect is dose-dependent, clearly noticeable with GM-CSF concentration as low as 0.5 nM.

Fig. 4 shows that the increase in both phosphorylation and enzyme activity elicited by GM-CSF are time-dependent. A maximum phosphorylation at 3 min is seen for phospho-Thr^{421}/Ser^{424} (Fig. 4*A*) as well as for phospho-Thr^{389} (Fig. 4*B*). Phosphorylation of Thr^{389} is seen as an increase in density of the immunoprecipitated p70 band. The Thr^{421}/Ser^{424} dual phosphorylation is seen at 3–5 min post GM-CSF, and is demonstrated by both a robust increase in density of the immunoprecipitated p70 band and by an upward mobility shift (Fig. 4*A*). The figure also shows results of immunoprecipitation with anti-p70S6K antibody and immunoblotting with the same antibody to demonstrate equal loading (Fig. 4*C*). *In vitro* kinase activity experiments also reveal a time-dependent increase due to GM-CSF (Fig. 4*D*). Maximal activity is reached at 5 min and declines slightly thereafter, and as such, the biphasic pattern of phosphorylation seen in Fig. 4, *A* and *B* correlated with enzymatic activity.

Thus, the results presented in Figs. 1–4 demonstrate that p70S6K is expressed in human neutrophils, that GM-CSF increased its enzymatic activity in a time and dose-dependent fashion, changes the V_{max} for its peptide substrate, and that this cytokine induces robust phosphorylation in 3 key residues: Thr^{389}, Thr^{421}, and Ser^{424}.

mTOR Is Involved in GM-CSF-activated p70S6K in Neutrophils—The next series of experiments were aimed at investigating what the mechanism that accounts for the observed increases in both phosphorylation and activity, was. Our first approach was the use of the immunosuppressant drug rapamycin, a well known inhibitor of mTOR, one of the several upstream regulators of p70S6K (17). In neutrophils, rapamycin inhibited GM-CSF-stimulated p70S6K Thr^{389} phosphorylation in a concentration-dependent manner (Fig. 5*A*). The intensity of phospho-Thr^{389} signal all but disappears at 10 nM.

The effect of rapamycin on the dual phosphorylation Thr^{421}/Ser^{424} was also very profound, but manifested itself differently and warranted close examination. Fig. 6*A* shows that rapamycin causes a dramatic downward mobility shift in the phospho-Thr^{421}/Ser^{424} band observed in the presence of GM-CSF. In this study, for the sake of clarification, we have labeled the lower band in GM-CSF + rapamycin as *a* and the upper band in GM-CSF alone as *b*. A shift from the upper *b* to lower *a* band observed with rapamycin treatment, is consistent with p70S6K dephosphorylation, as a less phosphorylated species runs faster in SDS-PAGE. Also shown in Fig. 6*A* (to the *right*) is a representative Western blot of fibroblast lysates to provide yet one more relative mobility comparison. The upper *b* band in GM-CSF-treated neutrophils has a M_r similar to that of serum-treated fibroblasts. Fig. 6*A* also shows that, in contrast to what was observed with phospho-Thr^{389} where the intensity signal disappeared with rapamycin + GM-CSF (Fig. 5*A*), a positive signal is still present to anti-phospho-Thr^{421}/Ser^{424} antibodies, indicating that phosphorylation of Thr^{421}/Ser^{424} was not completely affected by rapamycin.

To ascertain if the signal to Thr^{421}/Ser^{424} antibodies still remained simply because there was not enough rapamycin to inhibit it, we analyzed the status of this dual phosphorylation in response to GM-CSF with a range of rapamycin concentrations. Fig. 6*B* confirms that concentrations of rapamycin as low as 0.5 nM had already increased the mobility of p70S6K, and this effect remains present at all concentrations tested. A

FIG. 2. **Biochemical characterization of neutrophil p70S6K.** Neutrophils were challenged with GM-CSF and treated as indicated above. The kinase assay was performed against the KKRNRTLTK (*p70S6K peptide substrate*). Activity was measured as a function of incubation time of the *in vitro* enzyme assay (*A*); protein concentration (*B*), and substrate concentration at 0.5–0.7 mg/ml protein (*C*). From *C*, a Lineweaver-Burk plot was derived (*D*) from which the V_{max} and K_m values were calculated.

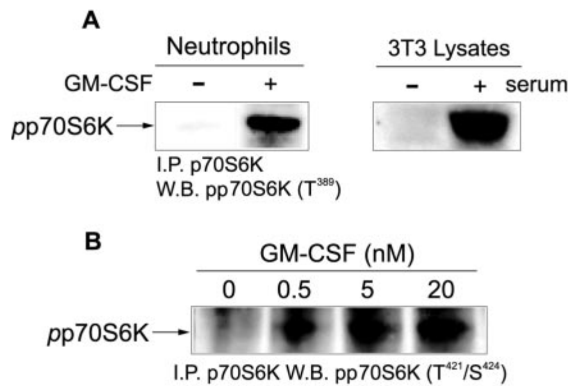

FIG. 3. **Phosphorylated species of p70S6K in GM-CSF-stimulated neutrophils.** *A*, presence of phospho(Thr^{389})p70S6K species. Neutrophils were stimulated with (+) or without (−) GM-CSF for 3 min, and lysates were prepared in boiling SDS buffer, followed by immunoprecipitation (*I.P.*) with anti-p70S6K and analyzed by Western blotting (*W.B.*) with anti-phospho(Thr^{389})p70S6K antibodies. As controls, lysates of NIH 3T3 fibroblast previously stimulated with (+) or without (−) serum, were run in parallel and probed with the same antibody. *B*, presence of phospho(Thr^{421}/Ser^{424})-p70S6K species: dose response with GM-CSF. Cells were stimulated with the indicated GM-CSF concentrations for 3 min, and samples were prepared as above.

weakened signal to the antibody was noticeable at higher concentrations (with similar protein loading in the SDS gel, as shown in Fig. 6*B*), but the signal was not completely eliminated, even at a concentration of 50 nM. Thus, rapamycin causes a potent change in electrophoretic mobility of p70S6K but there was still a rapamycin-resistant component of phosphorylation.

A partial inhibition of p70S6K by rapamycin is not entirely unlikely. Although it was originally described as a blocker of agonist-stimulated p70S6K activity in several cell lines (11, 29, 31), rapamycin may have differential effects on other systems. A dissociation between TOR kinase activity and rapamycin action has been already described (40, 41). Additionally, a recent example of discrepancy between rapamycin inhibition and p70S6K activity could be found in (42). At any rate, an inhibitor of a S6K cellular pool should be enough to explain the profound physiological effect of rapamycin on chemotaxis.[3]

We needed to prove that the mobility downshift from *b* to *a* is indeed due to the disappearance of phosphorylated residues. For this, we performed the following control experiment: we treated GM-CSF-stimulated neutrophil lysates with increasing concentrations of alkaline phosphatase *in vitro*. Then, we used the lysates to perform p70S6K immunoprecipitation and immunoblotting in a fashion similar to that followed in the previous experiments. Fig. 6*C* shows that as the phosphatase concentration increases for 10–80 units/ml, the phosphorylated p70S6K band shifts from the original *b* position (GM-CSF-stimulated cells, no alkaline phosphatase added) to the lower *a*

[3] J. Gomez-Cambronero, unpublished observations.

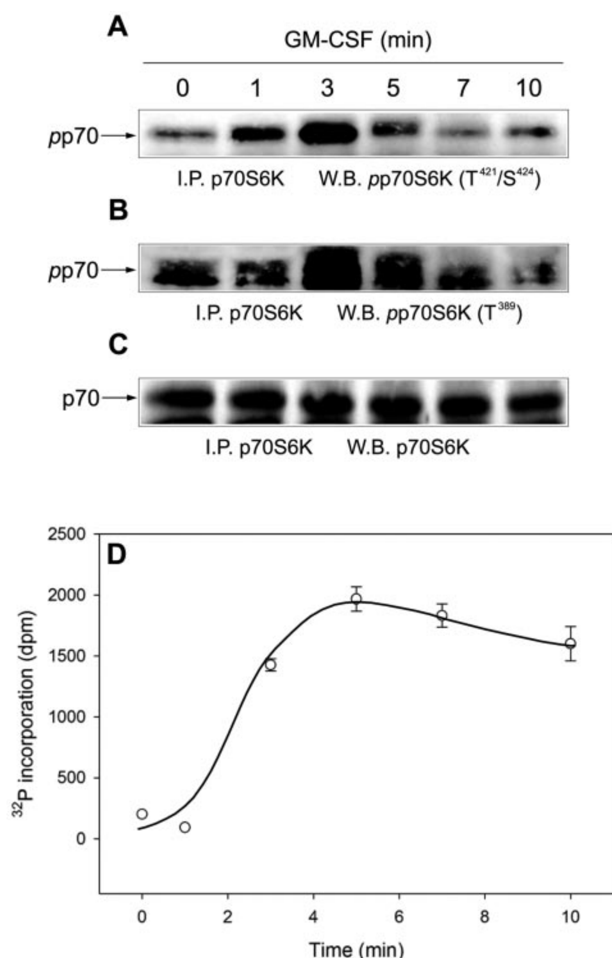

FIG. 4. **Time course of p70S6K activation.** Neutrophils were stimulated with 10 nM GM-CSF for the indicated lengths of time. Cell lysates were obtained, immunoprecipitated with anti-p70S6K antibodies, and immunocomplex beads were utilized for Western blotting with the indicated antibodies (*A–C*) or for assaying p70S6K enzymatic activity *in vitro* (*D*). *Panel C* confirms that protein loading is similar and that the small differences in protein per lane (kept at <5% by measuring protein in samples by Bradford assay before loading) can not account for differences in phosphorylation.

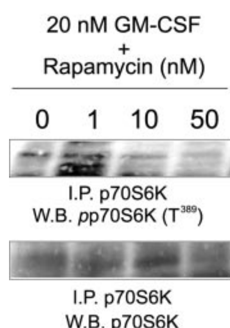

FIG. 5. **Effect of rapamycin on GM-CSF-stimulated p70S6K Thr^{389} phosphorylation.** Neutrophils were incubated with the indicated concentrations of rapamycin for 45 min, followed by a short (3 min) incubation with 10 nM GM-CSF. Shown are Western blots of anti-p70S6K immunoprecipitates probed with anti-phospho-Thr^{389}-p70S6K and with anti-p70S6K antibodies to show equal protein loading.

position. This position is equivalent to the rapamycin-treated GM-CSF-stimulated cells (Fig. 6, *A* and *B*). We therefore concluded that a shift of the nature observed in *p*p70S6K with rapamycin is, in effect, due to the loss of phosphate. Further,

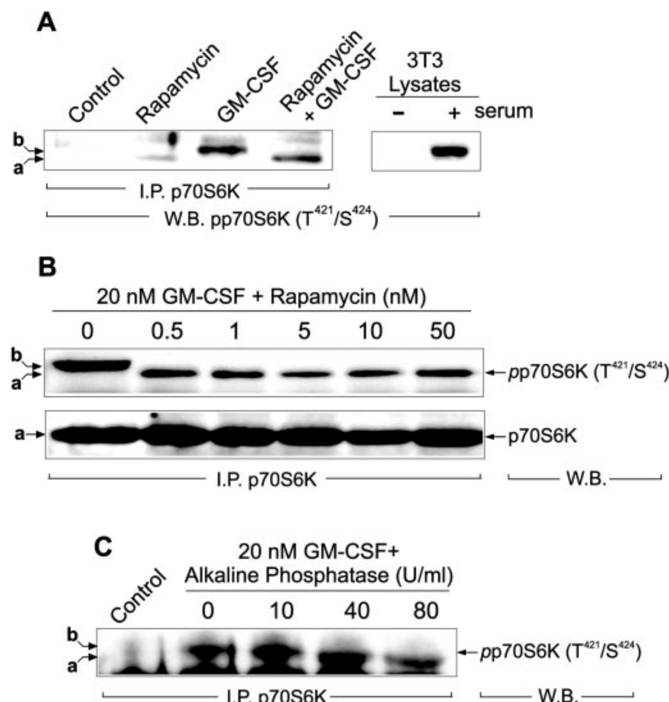

FIG. 6. **Effect of rapamycin on GM-CSF-stimulated p70S6K Thr^{421}/Ser^{424} phosphorylation.** *A*, neutrophils were incubated with 1 nM rapamycin for 45 min, followed by a short (3 min) incubation with 10 nM GM-CSF and cell lysates in boiling SDS were generated and immunoprecipitation with anti-p70S6K followed. Resulting immunocomplexes were used for immunoblotting with anti-phospho-Thr^{421}/Ser^{424}-p70S6K antibodies. As controls, lysates of NIH3T3 fibroblasts previously stimulated with (+) or without (−) serum, were run in parallel and probed with the same antibody. *Arrows* on the *left* show the relative mobility of less phosphorylated (*a*) and hyperphosphorylated (*b*) p70S6K species (see text for details). *B*, dose response with rapamycin. Neutrophils were incubated with the indicated concentrations of rapamycin for 45 min, and then challenged for 3 min with GM-CSF. Samples were processed as above. Anti-p70S6K immunoprecipitates were run in SDS gels and Western blots were probed with anti-Thr^{421}/Ser^{424}-p70S6K. After films were obtained, the blots were stripped of the antibody with a SDS/β-mercaptoethanol solution at 50 °C for 15 min, and they were then probed with anti-p70S6K antibodies, to show equal protein loading in all lanes and to confirm that the small differences in protein per lane (kept at <5% by measuring protein in samples by Bradford assay before loading) can not account for differences in phosphorylation. *C*, dephosphorylation by alkaline phosphatase. Neutrophils were treated as in *A*, and lysates were mixed with alkaline phosphatase at the indicated concentrations at a pH of 10. After phosphatase action, samples were immunoprecipitated and Western blots were probed with anti-phospho-Thr^{421}/Ser^{424} antibodies.

the maximum dose of alkaline phosphatase used in this experiment (80 units/ml), provides, in addition to the band downshift, a weakened signal to the antibodies (Fig. 6*C*), most likely due to the fact that alkaline phosphatase can dephosphorylate all the residues of a target substrate, regardless of the amino acid moiety.

The fact that rapamycin has a profound effect on changing the phosphorylation of p70S6K induced by GM-CSF points strongly to a participation of mTOR in the mechanism of signaling action. To further confirm this, we made use of FK506, another macrolide immunosuppressant structurally related to rapamycin. Like rapamycin, FK506 is a ligand of the immunophilin FK506-binding protein-12 (FKBP12) but exerts its action by mechanisms different from rapamycin (31). The mechanism is mTOR-related because: (*a*) unlike rapamycin, FK506 did not negatively affect the status of phosphorylation of p70S6K nor did it produce any downshift (Fig. 7*A*), and (*b*) the inhibition of GM-CSF-induced p70S6K phosphorylation caused by rapamycin was rescued by pretreatment of cells with FK506

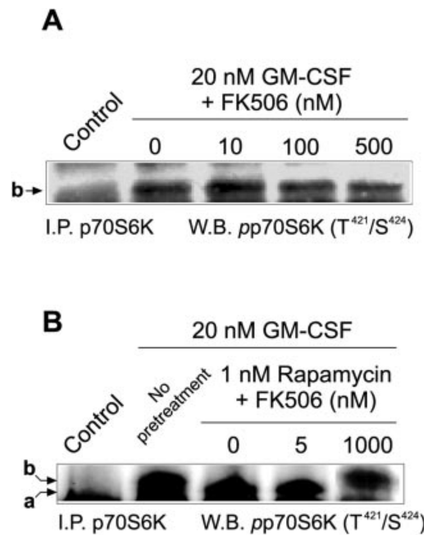

FIG. 7. **While FK506 does not inhibit p70S6K, it rescues rapamycin-induced inhibition.** *A*, dose response of FK506: Thr[421]/Ser[424] phosphorylation. Neutrophils were incubated with the indicated concentrations of FK506 for 30 min followed by a 3 min challenge with 10 nM GM-CSF. Cell lysates were generated that were used for immunoblotting with the indicated antibodies. *B*, rescue of rapamycin inhibition by FK506: Thr[421]/Ser[424] phosphorylation. Cells were preincubated with the indicated concentrations of FK506 for 30 min, and with rapamycin for another 30 min, followed by a final treatment with GM-CSF for 3 min. Cell lysates were generated and anti-p70S6K immunoprecipitated. Immunocomplex beads were used for immunoblotting with anti-phospho(Thr[421]/Ser[424])p70S6K antibodies. *No pretreatment* represents cells that did not receive FK506 or rapamycin but were stimulated with GM-CSF.

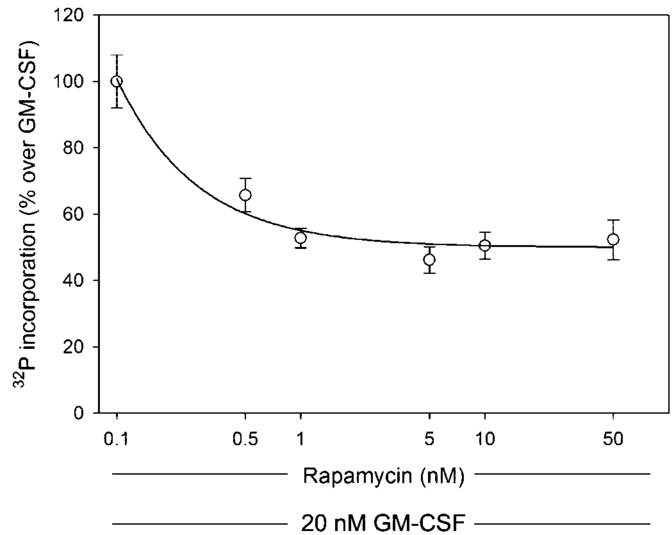

FIG. 8. **Effect of rapamycin on GM-CSF-stimulated p70S6K activity.** Neutrophils were incubated with the indicated concentrations of rapamycin for 45 min, followed by a short (3 min) incubation with 10 nM GM-CSF. Anti-p70S6K immunoprecipitates were produced and used for assaying ribosomal S6K enzymatic activity *in vitro*. 100% represents 2951 ± 264 dpm in the presence of GM-CSF alone.

(Fig. 7*B*). The less phosphorylated *a* band moves up to restore the mobility of the hyperphosphorylated *b* band at 1 μM FK506. This high concentration is similar to that used by other authors in this type of experiments (17, 31). Since FK506 binds FKBP12, which is also the soluble receptor for rapamycin, a competition between the two ligands ensues. It has been established earlier that at high enough concentrations, FK506 reverts the effects induced by rapamycin (17, 31, 43–45).

MAPK Is the Other Necessary Component Involved in GM-CSF-activated p70S6K in Neutrophils—Since rapamycin could not completely dephosphorylate p70S6K, we were interested in knowing at what extent the *in vitro* enzymatic activity of p70S6K would be affected by rapamycin. As presented in Fig. 8, pretreatment of cells with rapamycin prior to stimulating with GM-CSF caused an inhibition of enzymatic activity with an IC_{50} of 0.2 nM. However, this inhibition was never complete but, instead, reached only 40–50% of the GM-CSF-induced maximal level. Higher doses of rapamycin, even as high as 50 nM, did not cause further inhibition. These results indicate that neutrophil p70S6K activity, similarly to phosphorylation, has a rapamycin-resistant component. To investigate what the regulation of this component might be, we based our next experiments on the previous observation from our laboratory that there is a cross-talk between the MAPK and the p70S6K pathway (36), as well as on other authors work (46) who have indicated that a cluster of Ser/Thr-Pro sites in the p70S6K C-terminal tail can be phosphorylated *in vitro* by proline-directed kinases, such as ERK1/2 or cdc2.

We were wondering if the MEK/MAPK pathway could account for the rapamycin-resistant component of S6K activation. As presented in Fig. 9*A*, the MEK inhibitor PD-98059 (MEKi) also caused a partial inhibition of p70S6K activity. However, when cells were treated with a combination of PD-98059 and rapamycin prior to stimulation with GM-CSF, the loss of activity was complete, with levels returning to basal levels (note that *bars* on the *far right* and the *far left* are about equal). As an additional proof of the cooperative inhibitory effect of the combination rapamycin + MEKi, we measured F-actin polymerization by flow cytometry. Results in Table I show that even though the effects of rapamycin and MEKi on inhibiting actin were mild, when they were combined the levels of actin returned to basal, pre-stimulatory levels.

Next, in order to show that not just any combination of rapamycin with another drug would further the inhibition of p70S6K, we tested a combination of rapamycin and FK506. As seen in Fig. 9*B*, this combination nor only did not improve the inhibitory effect of rapamycin but, rather, enhanced it (*in vitro* kinase activity of GM-CSF-treated neutrophils is not inhibited with FK506, not shown).

Finally, Fig. 10 shows that at the level of phosphorylation, a combination of rapamycin and MEKi produced a downshift in the phospho band and diminished signal to the anti-phospho antibodies. Fig. 10*A* shows results with phospho-Thr[421]/Ser[424], and Fig. 10*B* with phospho-Thr[389] very similar to that observed with the *in vitro* treatment with alkaline phosphatase of Fig. 6*C*, that produced a large removal of phosphate. It should be noted that the dephosphorylation is complete when the antibodies used were against Thr[389], which lends strength to the point of the full effect of the combination rapamycin plus MEKi.

DISCUSSION

We report here for the first time the presence of p70S6K in neutrophils (protein, activity and phosphorylation) and its modulation by the hematopoietic growth factor GM-CSF. The data obtained in neutrophils become extremely important in view of the fact that these cells are terminally differentiated. As such, their translation machinery is not very active, yet the kinase that is supposed to be crucial in translation (ribosomal p70S6K) is present and highly activable by nanomolar concentrations of a physiological cytokine. In this study, we have also established the mechanism of p70S6K activation as involving two other cell signaling kinases. Phosphorylation of either Thr[389] or Thr[421]/Ser[424] on p70S6K is necessary but not sufficient to achieve full activation. Both have to be present during cell stimulation.

As far as we can discern, this is the first full report showing

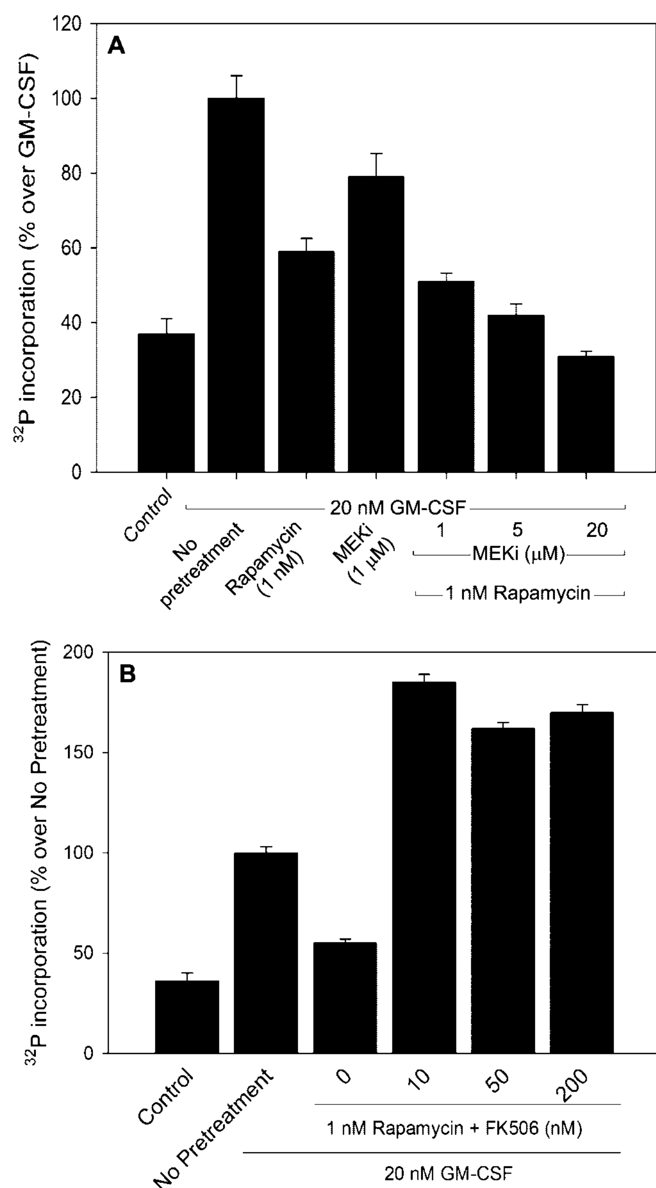

FIG. 9. **Full blockage of GM-CSF-stimulated p70S6K activity by a PD-98059 and rapamycin combination.** *A*, neutrophils were incubated with the indicated concentrations of rapamycin or PD-98059 (*MEKi*) for 30 min followed by a 3 min challenge with 10 nM GM-CSF. Cell lysates were generated that were immunoprecipitated with anti-p70S6K antibodies and immunocomplexes were used for phosphorylation immunoblotting. *Panel B* shows that the combination effect is specific for MEKi and rapamycin, since FK506 and rapamycin caused an activation. Cells were preincubated with the indicated concentrations of FK506 for 30 min, and with rapamycin for another 30 min, followed by a final treatment with GM-CSF for 3 min. Cell lysates were generated and anti-p70S6K immunoprecipitated. Immunocomplex beads were used for *in vitro* kinase activity. 100% represents 3420 ± 659 dpm in the presence of GM-CSF alone.

TABLE I
Flow cytometry analysis of actin polymerization in response to p70S6K modulators

Neutrophils were incubated with inhibitors for 45 minutes and then challenged with 10 nM GM-CSF at 37°C for 5 min. The stimulation was stopped by fixing the cells in formaldehyde. 200-μl samples of 1×10^4 cells were incubated with phalloidin-FITC and analyzed by flow cytometry. The log-fluorescence in each experiment was derived from Cell Quest software analysis. Results are the mean ± S.E. of three independent experiments.

	Log fluorescence intensity	
	No addition	GM-CSF
Control	3.04 ± 0.25	5.67 ± 0.37
Rapamycin 5 nM	3.65 ± 0.20	4.81 ± 0.31
MEKi 10 μM	3.16 ± 0.19	4.74 ± 0.39
Rapamycin + MEKi	3.20 ± 0.24	3.58 ± 0.22

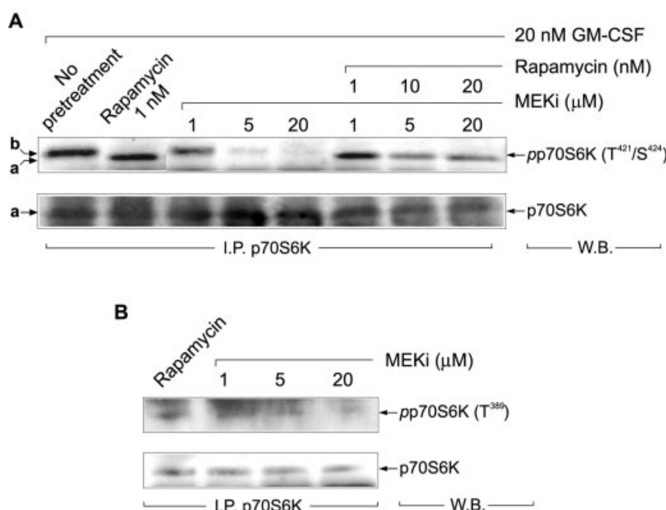

FIG. 10. **The combination of PD-98059 and rapamycin fully inhibits GM-CSF-stimulated p70S6K phosphorylation.** Neutrophils were incubated with the indicated concentrations of rapamycin or PD-98059 (*MEKi*), or their combination, for 30 min followed by a 3 min challenge with 10 nM GM-CSF. Cell lysates were generated that were immunoprecipitated with anti-p70S6K antibodies and immunocomplexes were used for phosphorylation immunoblotting with either anti-Thr^{421}/Ser^{424}phospho70S6K (*A*) or anti-Thr^{389} phospho70S6K (*B*). Anti-p70S6K controls are also included to show equal protein loading.

p70S6K in neutrophils and, for that matter, in a hematopoietic primary cell, its kinetic data, and its mechanism of regulation with GM-CSF. The enzyme velocity value for neutrophils (7.2 to 20.5 pmol/min/mg protein) of Fig. 2*D* is well in agreement with other authors who have reported kinetic data in the low picomolar range for hepatoma cells, PMA-stimulated, transfected COS-7, liver extracts of cycloheximide-stimulated rats and ion-exchange purified cell extracts, Swiss 3T3 cells stimulated with EGF and insulin and *Xenopus* oocytes (11, 47–51). Only the enzyme purified to near homogeneity displays activity in the high nanomolar range (47, 50, 51). There are two precedents that have briefly touched upon the presence of p70S6K in either leukemic cells or mature phagocytes. In a study of the role of STAT3 in GM-CSF-induced enhancement of neutrophilic differentiation of Me_2SO-treated HL-60 cells, Yamaguchi *et al.* (52) found that p70S6K could be activated. p70S6K is also activated upon PECAM-1/CD31 cross-linking, based on the appearance of serine phosphorylation in S6K immunoprecipitates (53). However, since p70S6K is not directly involved in integrin function (as per rapamycin evidence) the authors emphasize the upstream link, PI3K, as a common pathway of integrin and adhesiveness regulation in leukocytes.

To investigate p70S6K in neutrophils, we have concentrated in a parallel study of enzyme activity and analysis of the phosphorylation status of three key residues (Thr^{389} and Thr^{421}/Ser^{424}), working with specific anti-p70S6K immunoprecipitates. Immunoprecipitation with specific anti-p70S6K antibodies and immunocomplex assays with an also specific p70S6K peptide substrate (KKRNRTLTK, that bears the consensus site phosphorylation), accurately describes this particular kinase. Phosphorylation of 40 S ribosomal subunits (a natural substrate bearing the S6 protein) as presented in Fig. 1*B* also serves to fully confirm the p70S6K activity in neutrophils. To study the mechanism of p70S6K regulation elicited by GM-CSF in neutrophils, we have employed several strategies,

the first one being the use of rapamycin. We have observed that this immunosuppresant does inhibit Thr^{389} phosphorylation as previously reported by other authors (44). These authors have established that a mutation of Thr^{389} to alanine (T389A) makes p70S6K rapamicyn resistant and "kinase dead," *i.e.* the enzyme becomes unable to be activated by cytokines or growth factors. However, an endogenous, native, kinase like the one described here in neutrophils can be partially activated by GM-CSF even in the presence of rapamycin, since other residues will be available for phosphorylation in the intact cells. Furthermore, the same authors mutated Thr^{389} to an acidic residue and the T389E mutant in quiescent cells had a high basal activity and was able to be activated by serum, but only to a 50% level of the wild-type construct (44). Data in Fig. 8 (present paper) seem to be in agreement with results on the T389E mutant, but not with T389A. Also regarding rapamycin in the present study, but this time as we investigated residues other than Thr^{389}, we uncovered for the first time the unexpected effect of rapamycin on mobility shifts on Thr^{421}/Ser^{424}. The effect was very dramatic as it represented a change of ~3–4 kDa in the gels (Fig. 6, *A* and *B*). This correlated well with a loss of phosphate as presented in control experiments with alkaline phosphatase (Fig. 6*C*). The origin of this phosphate could not be established at this time but since the anti-phospho-Thr^{421}/Ser^{424} antibodies still gave a positive signal, it could not be associated to Thr^{421}/Ser^{424} residues but to any of the several others that have been reported to be phosphorylated upon growth factor or cytokine stimulation (3, 12, 13, 14).

Another strategy to study the mechanism of p70S6K activation, has been the use of FK506. The rapamycin-related, immunophilin, FK506 does not inhibit mTOR or p70S6K (rather, it acts through PP2A). As such, it is normally used in these type of studies as a valuable test to ascertain if an agonist effect specifically utilizes mTOR/p70S6K as a cell signaling mechanism. Our data show that FK506 does not inhibit, indicating that GM-CSF does work through mTOR/p70S6K. But the use of FK506 had an additional benefit. Since this drug binds to the FKBP12, which happens to be also the soluble receptor for rapamycin, a competition between the ligands ensue. At high enough concentrations, FK506 reverts the effects of rapamycin. Even though this has been reported earlier (17, 31) the present article is the first of its kind to have uncovered this in neutrophils, and further proves that point. Also here, the novel finding of rapamycin inhibiting only 40–50% of the enzymatic activity: the remainder is inhibited when the MEK/MAPK pathway is altered.

Since the combination of rapamycin and MEKi is necessary for full inhibition of p70S6K activity and phosphorylation, we can reasonably conclude that the two known kinases targeted by these inhibitors are needed to activate p70S6K in the first place. A model for the activation of p70S6K in human neutrophils is presented in Fig. 11. The data in this study are consistent with GM-CSF dually stimulating the activity of a Thr^{421}/Ser^{424} kinase and a Thr^{389} kinase, resulting in full activation of neutrophil ribosomal p70S6K. Although the identity of these two kinases has not been addressed in this study, the data strongly point to a MEK-related or -activated Thr^{421}/Ser^{424} kinase (possibly MAPK) and a rapamycin-sensitive, mTOR-related or -activated Thr^{389} kinase (possibly TOR itself). This study also indicates that both pathways must work simultaneously in order to achieve full activation of p70S6K by GM-CSF.

The effects of rapamycin could be mediated by either blocking the activation of a Thr^{389} kinase or by activating a Thr^{389} phosphatase, or by a combination of both. Also, other residues that we have not explored in this study might be implicated. Even though it is well known (3, 4, 12, 13, 17–21) that at least

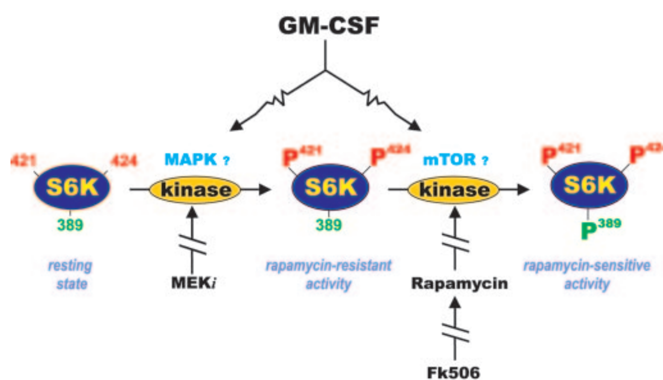

FIG. 11. **Model of the activation of p70S6K in human neutrophils.** Incubation of human neutrophils with GM-CSF results in the dual activation of a Thr^{421}/Ser^{424} kinase and a Thr^{389} kinase. Data presented here point to a MEK-related, Thr^{421}/Ser^{424} kinase (possibly MAPK), and a rapamycin-sensitive, mTOR-related (possibly TOR itself), Thr^{389} kinase. Rapamycin could either inactivate the second kinase activity or activate a phosphatase. This effect is reversed by FK506. Additionally, p70S6K receives a positive input from the MEK/MAPK pathway. The latter point has also been demonstrated by Lehman and Gomez-Cambronero (36) by co-immunoprecipitation analysis.

12 Ser/Thr residues can be phosphorylated upon cell stimulation, there would be unpractical to examine each one of them. Importantly, Ser^{371} is a critical residue (13) that could explain that phosphorylation of Thr^{389} or Thr^{421}/Ser^{424} is necessary but not sufficient to achieve full activation. Also, more experiments are needed to verify if MAPK signaling indeed only regulates S6 kinase via its C-terminal site. All and all the further unveiling of the molecular mechanism of GM-CSF-induced p70S6K activation in neutrophils deserves future investigation. A confirmation of the results presented here could come from the study of mammalian cell transfection with DNA mutants (which will be technically challenging in the neutrophil, a short-lived cell that can not be cultured) or with protein mutants. To this respect, Gardiner *et al.* (54) have shown that protein delivery (or transduction) to neutrophils can be successfully accomplished, as demonstrated with *Rac*. However tantalizing these kind of reconstitution studies may be, the lack of crucial molecular reagents (the full-length, wild-type p70S6K protein, and dominant negative or constitutively active mutants) prevent further studies on p70S6K at this time.

At any event, results shown here with neutrophils clearly indicate for the first time that we should look for effects of p70S6K other than those described for the ribosomal machinery, since the translational capabilities (the role classically assigned to p70S6K as an activator of ribosomal protein S6) of those cells are minimal. To this respect, other authors have proposed that p70S6K is involved in IL-8 production in mononuclear cells from rheumatoid arthritis synovial tissue (55). In macrophages, p70S6K has been linked with FKBP12-rapamycin-associated phosphorylation of iNOS and LPS-induced NO production and TNF-a synthesis (56, 57). A role of p70S6K in survival of cytokine-stimulated T cells or apoptosis has been ruled out (32). As an extension of the present study, we are currently exploring a possible role for p70S6K in one key physiological response of neutrophil during phagocytosis, cell migration, that is in agreement with cytoskeletal polymerization data presented in Table I.

Acknowledgments—We thank Dr. Mauricio Di Fulvio for helpful suggestions and excellent artwork, Jeff Horn for help with the phosphatase assay, and Dr. Patrick Dennis for excellent insights on the discussion

REFERENCES

1. Nemenoff, R. A., Price, D. J., Mendelsohn, M. J., Carter, E. A., and Avruch, J. (1988) *J. Biol. Chem.* **263,** 19455–19460
2. Proud, C. G. (1996) *Trends Biochem. Sci.* **21,** 181–185
3. Pullen, N., and Thomas, G. (1997) *FEBS Lett.* **410,** 78–82
4. Kawasome, H., Papst, P., Webb, S., Keller, G. M., Johnson, G. L., and Gelfand, E. W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**
5. Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C., and Thomas, G. (1999) *Science* **285**
6. Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Kozma, S. C. (1998) *EMBO J.* **17,** 6649–6659
7. Grove, J. R., Banerjee, P., Balasubramanyam, A., Coffer, P. J., Price, D. J., Avruch, J., and Woodgett, J. R. (1991) *Mol. Cell. Biol.* **11,** 5541–5550
8. Reinhard, C., Fernandez, A., Lamb, N. J., and Thomas, G. (1994) *EMBO J.* **13,** 1557–1565
9. Gout, I., Minami, T., Hara, K., Tsujishita, Y., Filonenko, V., Waterfield, M. D., and Yonezawa, K. (1998) *J. Biol. Chem.* **273,** 30061–30064
10. Lee-Fruman, K. K., Kuo, C. J., Lippincott, J., Terada, N., and Blenis, J. (1999) *Oncogene* **18,** 5108–5114
11. Price, D. J., Grove, J. R., Calvo, V., Avruch, J., and Bierer, B. E. (1992) *Science* **257,** 973–977
12. Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H., and Sabatini, D. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1432–1437
13. Saitoh, M., Pullen, N., Brennan, P., Cantrell, D., Dennis, P. B., and Thomas, G. (2002) *J. Biol. Chem.* **227,** 20104–20112
14. Weng, Q. P., Kozlowski, M., Belham, C., Zhang, A., Comb, M. J., and Avruch, J. (1998) *J. Biol. Chem.* **273,** 16621–16629
15. Avruch, J., Belham, C., Weng, Q., Hara, K., and Yonezawa, K. (2001) *Prog Mol. Cell. Biol.* **26,** 115–154
16. Dufner, A., and Thomas, G. (1999) *Exp. Cell Res.* **253,** 100–109
17. Han, J. W., Pearson, R. B., Dennis, P. B., and Thomas, G. (1995) *J. Biol. Chem.* **270,** 21396–21403
18. Balendran, A., Currie, R., Armstrong, C. G., Avruch, J., and Alessi, D. R. (1999) *J. Biol. Chem.* **274,** 37400–37406
19. Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N., and Avruch, J. (1998) *Curr. Biol.* **8,** 69–81
20. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. (1998) *Science* **279,** 707–710
21. Belham, C., Comb, M. J., and Avruch, J. (2001) *Curr. Biol.* **11,** 1155–1167
22. Isotani, S., Hara, K., Tokunaga, C., Inoue, H., Avruch, J., and Yonezawa, K. (1999) *J. Biol. Chem.* **274,** 34493–34498
23. Romanelli, A., Dreisbach, V. C., and Blenis, J. (2002) *J. Biol. Chem.* **277,** 40281–40289
24. Gonzalez-Garcia, A., Garrido, E., Hernandez, C., Alvarez, B., Jimenez, C., Cantrell, D. A., Pullen, N., and Carrera, A. C. (2002) *J. Biol. Chem.* **277,** 1500–1508
25. Calvo, V., Crews, C. M., Vik, T. A., and Bierer, B. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7571–7575
26. Calvo, V., Wood, M., Gjerston, C., Vik, T., and Bierer, B. E. (1994) *Eur. J. Immunol.* **24,** 2664–2671
27. Reif, K., Burgering, B. M. T., and Kantrell, A. (1997) *J. Biol. Chem.* **272,** 14426–14433
28. Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B., and Schreiber, S. L. (1995) *Nature* **377,** 441–446
29. Kuo, C. J., Chung, J., Fiorentino, D. F., Flanagan, W. M., Blenis, J., and Crabtree, G. R. (1992) *Nature* **358,** 70–73
30. Lane, H. A., Fernandez, A., Lamb, N. J., and Thomas, G. (1993) *Nature* **363,** 170–172
31. Chung, J., Kuo, C. J., Crabtree, G. R., and Blennis, J. (1992) *Cell* **69,** 1227–1236
32. Scheid, M. P., Charlton, L., Pelech, S. L., and Duronio, V. (1996) *Biochem. Cell Biol.* **74,** 595–600
33. Gomez-Cambronero, J., and Veatch, C. (1996) *Life Sci.* **59,** 2099–2111
34. Hunter, M. G., and Avalos, B. R. (2000) *Blood* **95,** 2132–2137
35. Lehman, J. A., Paul, C. C., Baumann, M. A., and Gomez-Cambronero, J. (2001) *Am. J. Physiol. Cell Physiol.* **280,** 183–191
36. Lehman, J. A., and Gomez-Cambronero, J. (2002) *Biochem. Biophys. Res. Commun.* **293,** 463–469
37. English, D., and Andersen, B. R. (1974) *J. Immunol. Methods* **5,** 249–252
38. Joseph, D. E., Paul, C. C., Baumann, M. A., and Gomez-Cambronero, J. (1996) *J. Biol. Chem.* **271,** 13088–13093
39. Egger, G., Burda, A., and Glasner, A. (2001) *Virchows Arch.* **438,** 394–397
40. Zheng, X. F., Florentino, D., Chen, J., Crabtree, G. R., and Schreiber, S. L. (1995) *Cell* **82,** 121–130
41. Erbay, E., and Chen, J. (2001) *J. Biol. Chem.* **276,** 36079–36082
42. Patel, S., Lochhead, P. A., Rena, G., Fumagalli, S., Pende, M., Kozma, S. C., Thomas, G., and Sutherland, C. (2002) *J. Biol. Chem.* **277,** 9889–9895
43. Bierer, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G., and Schreiber, S. L. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9231–9235
44. Pearson, R. B., Dennis, P. B., Han, J. W., Williamson, N. A., Kozma, S. C., Wettenhall, R. E., and Thomas, G. (1995) *EMBO J.* **14,** 5279–5287
45. Dumont, F. J., Melino, M. R., Staruch, M. J., Koprak, S. L., Fischer, P. A., and Sigal, N. H. (1990) *J. Immunol.* **144,** 1418–1424
46. Mukhopadhyay, N. K., Price, D. J., Kyriakis, J. M., Pelech, S., Sanghera, J., and Avruch, J. (1992) *J. Biol. Chem.* **267,** 3325–3335
47. Price, D. J., Nemenoff, R. A., and Avruch, J. (1989) *J. Biol. Chem.* **264,** 13825–13833
48. Ahn, N. G., Weiel, J. E., Chan, C. P., and Krebs, E. G. (1990) *J. Biol. Chem.* **265,** 11487–11494
49. Cicirelli, M. F., Pelech, S. L., and Krebs, E. G. (1988) *J. Biol. Chem.* **263,** 2009–2019
50. Lane, H. A., and Thomas, G. (1991) *Methods Enzymol.* **200,** 268–291
51. Siegmann, M., and Thomas, G. (1987) *Methods Enzymol.* **146,** 362–369
52. Yamaguchi, T., Mukasa, T., Uchida, E., Kanayasu-Toyoda, T., and Hayakawa, T. (1999) *J. Biol. Chem.* **274,** 15575–15581
53. Pellegatta, F., Chierchia, S. L., and Zocchi, M. R. (1998) *J. Biol. Chem.* **273,** 27768–27771
54. Gardiner, E. M., Pestonjamasp, K. N., Bohl, B. P., Chamberlain, C., Hahn, K. M., and Bokoch, G. M. (2002) *Curr. Biol.* **12,** 2029–2034
55. Salh, B., Wagey, R., Marotta, A., Tao, J. S., and Pelech, S. (1998) *J. Immunol.* **161,** 6947–6954
56. Hayes, A. L., Smith, C., Foxwell, B. M., and Brennan, F. M. (1999) *J. Biol. Chem.* **274,** 33455–33461