

## S6 Kinase p90<sup>rsk</sup> in Granulocyte-Macrophage Colony-stimulating Factor-stimulated Proliferative and Mature Hematopoietic Cells\*

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**The ribosomal S6 kinase p90<sup>rsk</sup> was studied in mature and proliferating hemopoietic cells in response to the human cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF). In neutrophils, GM-CSF induced time-dependent electrophoretic mobility shifts in immunoreactive p90<sup>rsk</sup>. Although these shifts suggested changes in the phosphorylation status of the molecule, a kinase assay with whole cell lysates detected minimal (1.5-fold) increments in enzymatic activity. Only immunoprecipitation followed by immune complex kinase assay or in-gel kinase assay performed against the RSK substrate RRLSSLRA evidenced an increase in p90<sup>rsk</sup> activity (3.4-fold). p90<sup>rsk</sup> was also detected in the GM-CSF-dependent erythroleukemia cell line TF-1. Normally cultured, cytokine-supplemented cells did not respond to further GM-CSF stimulation. However, the activity of p90<sup>rsk</sup> in cytokine-starved cells increased dramatically in response to short term GM-CSF challenge. This effect was readily observable in total cell lysates (6.6-fold increase over controls) and was paralleled by changes in mitogen-activated protein kinase activity (a substrate of p90<sup>rsk</sup>). Thus, p90<sup>rsk</sup> is present in mature hemopoietic cells, but the extent of the enzymatic response to GM-CSF is significantly lower than that seen in proliferative cells.**

The granulocyte-macrophage colony-stimulating factor (GM-CSF),<sup>1</sup> like other related cytokines, has multiple functions in cells of the immune system, namely, proliferation, differentiation, end-cell activity, and regulation of apoptosis. GM-CSF, as a growth factor for immature hematopoietic cells, plays a crucial role in the commitment of bone marrow stem cells, directing the proliferation and differentiation into specific lineage precursors and enhancing their survival (1–3). Additionally, GM-CSF, as a stimulatory agent of terminally differentiated hematopoietic cells, is able to potentiate the activation of mature phagocytes, neutrophils, eosinophils, and macrophages, playing an important role in host defense (4). In the neutrophil, GM-CSF is able to enhance cell chemotaxis, antibody-dependent cell-mediated cytotoxicity, expression of membrane antigens, phagocytosis, degranulation of lytic enzymes, and release

of superoxide anions directed toward invading microbes (5).

In spite of lacking a tyrosine catalytic domain (6), the GM-CSF receptor is still able to induce rapid phosphorylation of multiple cell proteins on tyrosine residues in various cell types of the myeloid lineage, including the neutrophil (7–11). A few of these phosphoproteins have been identified so far, like p53/p56<sup>lyn</sup>, p62<sup>yes</sup>, and p92<sup>c-fes</sup> (12, 13). In addition, Ser/Thr kinases like c-RAF (14, 15), the  $\epsilon$  isoform of protein kinase C (16), PtdInsP<sub>2</sub>-specific phosphoinositol 3OH-kinase (17), JAK kinases/STAT, and mitogen-activated protein (MAP) kinases (18–22) have been involved in the signaling mechanisms of GM-CSF action. In fact, p42<sup>MAPK</sup> (ERK-2) is one of the proteins that undergoes the largest change in its tyrosyl phosphate content upon GM-CSF or fMet-Leu-Phe (chemotactic factor) treatment in the neutrophil.

As it has been previously shown, MAP kinases p42, p44, and p54, become active by dual Thr/Tyr phosphorylation in response to mitogenic stimuli (23, 24) and are able to phosphorylate other kinases downstream on Ser/Thr residues. Sturgill *et al.* (25) demonstrated that p42<sup>MAPK</sup> isolated from *Xenopus* oocytes was able to *in vitro* phosphorylate the ribosomal S6 kinase p90<sup>rsk</sup> in 3T3-L1 adipocytes. p90<sup>rsk</sup>, first named S6KII, was initially described in PC12 pheochromocytoma cells (26) and was purified from *Xenopus* eggs (27) as well as murine and avian cell lines (28, 29). It has been extensively studied in mitosis (30). p90<sup>rsk</sup> is itself a mitogen-activated Ser/Thr kinase. *In vitro* it can phosphorylate synthetic peptides like RRLSSLRA, which was modeled after the phosphorylation site of S6 protein of the mammalian small (40 S) ribosomal subunit (31, 32). Protein S6 has been long known to become the major phosphoprotein found in mammalian ribosomes of cells stimulated with growth factors (33). Phosphorylation of this protein, which occurs during mitogen and growth factor activation of cells, could facilitate protein synthesis (34, 35).

We undertook this study firstly to ascertain whether p90<sup>rsk</sup> was present in mature hematopoietic cells. Because we were able to detect a significant amount of p90<sup>rsk</sup> in neutrophils, we further studied whether the kinase enzymatic activity was regulated upon cell stimulation with GM-CSF and how this effect compared with the situation in proliferating hematopoietic cells.

### EXPERIMENTAL PROCEDURES

#### Materials, Antibodies, and Cell Lines

rhGM-CSF and rhTNF- $\alpha$  were purchased from R&D Systems (Minneapolis, MN); lipopolysaccharide was from Difco (Detroit, MI); fMet-Leu-Phe, PKA inhibitor (rabbit sequence) TTYADFIASGRTGRRNA-IHD, goat anti-rabbit and anti-mouse IgG (whole molecule agarose beads), and myelin basic protein were from Sigma; diisopropyl fluorophosphate was from Aldrich; Immobilion polyvinylidene difluoride membranes were from Millipore Corporation (Bedford, MA); electrophoresis chemicals were from Bio-Rad; [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) was from Amersham Life Sciences, Inc.; ion exchange chromatography cellulose

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<sup>1</sup> The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; MAP, mitogen-activated protein; FCS, fetal calf serum; FMLP, fMet-Leu-Phe; MBP, myelin basic protein.

phosphate paper was purchased from Whatman (Hillsboro, OR); anti-MAP kinase (p42, erk2), anti-pp90<sup>rsk</sup>, anti-GTPase-activating protein antibodies (directed against a peptide containing amino acid residues 171–448 of human GTPase-activating protein), RSK peptide (RRRLSS-LRA), and myelin basic protein (MBP) peptide (APRTGGRR) were from Upstate Biotechnology Inc. (Lake Placid, NY); the cervix epithelioid carcinoma HeLa cell lines were purchased from The American Type Culture Collection (Rockville, MD); the cytokine-dependent erythroleukemia cell line TF-1 was a generous gift of Dr. T. Kitamura, University of Tokyo, Japan; tissue culture material was from Becton Dickinson (Oxnard, CA) and Costar (Cambridge, MA).

#### Isolation of Human Neutrophils, Cell Culture, and Thymidine Incorporation

Human neutrophils were isolated from blood of healthy donors and separated on a Ficoll/Hypaque gradient according to the method of English and Anderson (36). TF-1 erythroleukemia cells were cultured as described previously (37). Briefly, cells were maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, and gentamicin (50 µg/ml) in the presence of GM-CSF (150 pM) at 37 °C, 5% CO<sub>2</sub> in a humidified atmosphere. Prior to some studies, the TF-1 cells were starved from cytokine maintenance by washing twice in RPMI 1640 supplemented with 2% FCS without GM-CSF. The cells were resuspended at 5 × 10<sup>4</sup> cells/ml in RPMI 1640 with 10% FCS and incubated for 48 h at 37 °C, 5% CO<sub>2</sub> in a humidified atmosphere. At the time of the experiment both cytokine-starved and control cytokine-supplemented populations were washed three times in RPMI. The proliferative ability of the TF-1 in response to cytokines was determined in [<sup>3</sup>H]thymidine incorporation studies. Briefly, starved TF-1 cells were washed once in RPMI 1640 containing 2% FCS and plated in 96-well plates at 2 × 10<sup>4</sup> cells/well in complete medium with or without cytokine, incubated for 48 h before a 6-h pulse with [<sup>3</sup>H]thymidine (0.5 µCi/well). The cells were then harvested, and the relative levels of the DNA-incorporated radioactivity was determined by liquid scintillation counting.

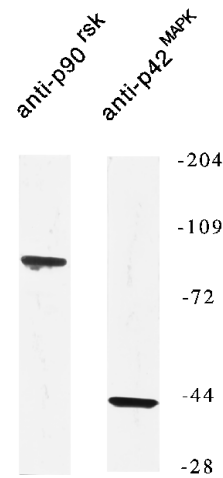
#### Preparation of Cell Extracts

At the time of the experiment, both TF-1 cells and peripheral circulating neutrophils were challenged with 270 pM GM-CSF. Cell suspensions were incubated with GM-CSF for various times at 37 °C and lysed by the addition of 10 volumes of the cell suspension to one volume of lysis buffer (0.1 M HEPES, pH 7.3, 0.7 mM sodium orthovanadate, 10 µM *p*-nitro-phenyl-phosphate, 10 mM EGTA, 5.5% Triton X-100, 0.5 M β-glycerophosphate, 10 µM phenylmethylsulfonyl fluoride, 0.1 mM ammonium molybdate, 12 mM diisopropyl fluorophosphate, 5 µg/ml leupeptin, 2 µg/ml aprotinin, and 7 µg/ml pepstatin A). Cell lysates (0.4–0.9 mg/ml protein) were incubated on ice for 20 min and then centrifuged for 5 min at 7,000 × *g*.

#### Kinase Enzymatic Assays

**<sup>32</sup>P Incorporation into Substrates from Whole Cell Lysates**—The enzymatic activities of p90<sup>rsk</sup> and p42<sup>MAPK</sup> were measured in whole cell lysates essentially as initially described (38) and later optimized for polymorphonuclear neutrophil cells (39). The RSK peptide (RRRLSS-LRA) was the phosphoacceptor substrate used for measuring p90<sup>rsk</sup> and the MBP peptide (APRTGGRR) was used for measuring p42<sup>MAPK</sup>. Either 100 µM RSK peptide, or 1 mM MBP peptide, was diluted in freshly prepared kinase buffer (13.4 mM HEPES, pH 7.3, 25 mM MgCl<sub>2</sub>, 30 mM Na<sub>2</sub>VO<sub>3</sub>, 5 mM *p*-nitrophenyl phosphate, 2 mM EGTA, 2 µM cAMP-dependent kinase inhibitor TTYADFIASGRTGRRNAIHD, 21 µCi of [<sup>32</sup>P]ATP/ml (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). This concentration is high enough, thus ensuring that protein kinase A is not utilizing RRRLSS-LRA as a substrate and will not mask the measurement of the p90<sup>rsk</sup> activity. To initiate the phosphotransferase reaction, aliquots (20 µl) of kinase buffer were mixed 1:1 (v/v) with the cell lysates. The reaction was carried out at room temperature for 10 min and terminated by blotting 20 µl of the reaction mixture onto P81 ion exchange chromatography cellulose phosphate papers. Filter squares were washed, dried, and counted for radioactivity.

**Immunoprecipitation and Immune Complex Kinase Assay**—Immunoprecipitation was carried out as reported previously (40) with some minor modifications that are described in detail as follows. The protocol entailed a first step of an overnight incubation of the primary antibody (anti-p90<sup>rsk</sup>) at a final concentration of 10 µg/ml with anti-rabbit (IgG, whole molecule) antibody conjugated to agarose beads in lysis buffer (see above for composition). The beads were thoroughly washed and



**FIG. 1. Detection of p90<sup>rsk</sup> and p42<sup>MAPK</sup> in human polymorphonuclear neutrophils.** Cell lysates were obtained from human polymorphonuclear neutrophils (1 × 10<sup>7</sup> cells/ml), and they were electrophoresed on SDS-polyacrylamide gel electrophoresis as indicated under "Experimental Procedures." Proteins were transferred onto polyvinylidene difluoride blots. These were probed with anti-p90<sup>rsk</sup> or anti-p42<sup>MAPK</sup> antibodies and developed by enhanced chemiluminescence. Molecular mass (in kDa) standards (from Life Technologies, Inc.) are indicated to the right of the figure. The figure shown is representative of four independent experiments with similar results.

then mixed with crude cell lysates prepared as indicated above at a ratio agarose beads/cell lysates 1:2 (v/v). After a 4-h incubation period at 4 °C, immune complexes were recovered by centrifugation. Pellets were washed two times with lysis buffer, two times with buffer A (100 mM Tris-HCl, pH 7.4, 400 mM LiCl) and two times with buffer B (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). The immunoprecipitation efficiency of both antibodies was monitored by Western blotting of the immunoprecipitates probed with the same kind of antibody used in the immunoprecipitation step. Immune complexes were resuspended in a final volume of 30 µl with lysis buffer and mixed with the kinase buffer. The reaction was carried out as indicated above.

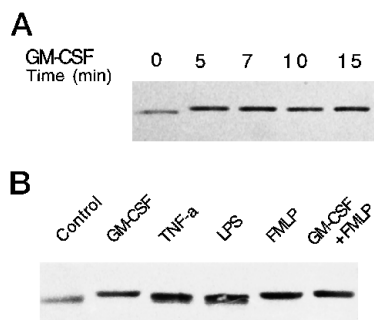
**In-gel Renatured Kinase Assay**—We followed the *in situ* or in-gel kinase assay as described previously (41). Briefly, either 50 µg/ml RRRLSS-LRA or 250 µg/ml MBP, to measure p90<sup>rsk</sup> or p42<sup>MAPK</sup>, respectively, was mixed with SDS-polyacrylamide gel electrophoresis gel solution before gel polymerization. After electrophoresis, gels were washed of SDS by incubation with 20% isopropanol in buffer A (100 mM Tris-HCl, pH 8, 5 mM 2-mercaptoethanol). Gels were then incubated with buffer A containing 6 M guanidine-HCl, and proteins were renatured by incubation at 4 °C for 6 h in buffer A containing 0.04% Tween 40. Renatured gels were overlaid with kinase buffer (20 mM Tris-HCl, pH 8, 10 mM MgCl<sub>2</sub>, 25 mCi of [<sup>32</sup>P]ATP) and incubated at room temperature for 30 min. Gels were extensively washed with 1% pyrophosphate in 5% trichloroacetic acid, dried, and exposed to x-ray films.

#### Western Blotting

Western blotting was performed as described previously (42). Cells were incubated with the appropriate stimulus and total cell lysates were prepared as indicated above. Proteins were electrophoresed and transferred to polyvinylidene difluoride. Blots were blocked in Tris-buffered saline-Tween-20 (TBS-T) (20 mM Tris-base, pH 7.6, 137 mM NaCl, 0.1% Tween 20) supplemented with 5% bovine serum albumin, followed by incubation with the required primary antibody (1:1,000 dilution) in TBS-T and finally with horseradish peroxidase-conjugated anti-mouse IgG (for anti-MAP kinase antibody) or anti-rabbit (for anti-p90<sup>rsk</sup> antibody) at a 1:7,000 dilution for 1 h at room temperature. Blots were autoradiographed for variable lengths of time (ranging from 15 s to 1 min) with Kodak x-Omat film. When needed, protein bands were quantified by laser scanning densitometry at 633 nm in a LKB Ultrascan (Pharmacia Biotech Inc.).

#### RESULTS

**Detection of p90<sup>rsk</sup> in the Human Neutrophil**—The presence of p90<sup>rsk</sup> could be readily detected in human neutrophils in Western blots developed with anti-p90<sup>rsk</sup> antibody (Fig. 1). This antibody recognizes a single band of 88 kDa, which corresponds



**FIG. 2. Effect of GM-CSF and neutrophil agonists on the relative mobility of p90<sup>rsk</sup>.** Neutrophils ( $1 \times 10^7$  cells/ml) were stimulated with 270 pM GM-CSF for the indicated lengths of time (A) or with 270 pM GM-CSF, 2 ng/ml tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), 50 ng/ml lipopolysaccharide plus 1% human serum, or 100 nM chemotactic factor FMLP for 5 min (B). In either case, cell lysates were obtained and Western blots were probed with anti-p90<sup>rsk</sup> antibodies. Shown in the figure are regions of the blots that span from 75 to 105 kDa. The figure shown is representative of four separate experiments.

closely to the reported molecular mass of p90<sup>rsk</sup>. One of the *in vitro* substrates of p90<sup>rsk</sup>, the MAP kinase isoform termed erk2 or p42<sup>MAPK</sup>, could also be detected in neutrophils (Fig. 1). Treatment of polymorphonuclear neutrophils with GM-CSF resulted in an upward shift in relative electrophoretic mobility of p90<sup>rsk</sup> (Fig. 2A). The observed electrophoretic mobility shifts were equivalent to an increment of approximately 4 kDa (gross quantitation based on the molecular weight standards' relative mobility) and could be observed after 5 min of incubation with GM-CSF. A slow migrating form was the predominant protein species in all the time points tested.

Less noticeable and more diffuse changes in electrophoretic mobility of p90<sup>rsk</sup> could be detected with TNF- $\alpha$  and lipopolysaccharide in the presence of 1% human serum (Fig. 2B). In contrast, the chemotactic factor fMet-Leu-Phe (FMLP) induced a mobility shift similar in magnitude to that exerted by GM-CSF. Moreover, when both stimulants are combined (GM-CSF preincubation of cells followed by FMLP challenge), the mobility shifts reached their maximum extension. An augmentation of an agonist-induced physiological response due to a cytokine is often referred to as "priming" (2). It could be possible that the maximum response observed in these studies with the combination of FMLP and GM-CSF falls into that category.

**GM-CSF and p90<sup>rsk</sup> Enzymatic Activity**—A mobility shift in immunoblots derived from SDS gels (Fig. 2) is indicative of a covalent modification of the protein, such as protein phosphorylation, that might lead to an increase in enzymatic activity. We first measured the enzymatic activity of p90<sup>rsk</sup> in whole cell lysates derived from control and GM-CSF-treated cells. Fig. 3A shows the results of the incorporation of <sup>32</sup>P onto the RSK peptide (RRRLSSLRA, which corresponds to amino acids 231–239 of human 40 S ribosomal protein S6). GM-CSF elicited a time-dependent activation of p90<sup>rsk</sup> that was only 1.5-fold over controls. Maximal responses were attained early (5–10 min). As a comparison, MAP kinase activity increased over 4-fold and peaked at 5 min after GM-CSF stimulation (Fig. 3B). A sharp return to basal levels was observed at later times that paralleled the changes in mobility shift of the molecule (Fig. 3B, *inset*). It could also be observed that the peak of p90<sup>rsk</sup> activity slightly lagged that of p42<sup>MAPK</sup>. The p42<sup>MAPK</sup> activation declined at a much higher slope than p90<sup>rsk</sup>.

We next examined whether the chemotactic factor FMLP, shown to induce a mobility shift in gels (Fig. 2), is also able to activate p90<sup>rsk</sup>. As demonstrated in Fig. 4A, FMLP produced only a minor (1.5-fold) increment in enzymatic activity. Only the combination of GM-CSF and FMLP provided a slightly higher increment in activity (2-fold over controls). The Ser/Thr-

specific protein phosphatases 1 and 2A inhibitor okadaic acid also had a positive effect on increasing the p90<sup>rsk</sup> activity, and genistein only marginally (30%) inhibited p90<sup>rsk</sup> activity. In contrast (Fig. 4B), MAP kinase activity was greatly increased by FMLP, and even more with combination FMLP + GM-CSF or with okadaic acid alone (up to 10-fold for the latter). Submicromolar concentrations of genistein inhibited (> 80%) the agonist-induced MAP kinase activation, as could be expected from a tyrosine kinase inhibitor.

**Immune Complex Kinase Assay of p90<sup>rsk</sup>**—It has been reported before (43) that kinase assays performed in total cell lysates sometimes do not allow the full activation of p90<sup>rsk</sup> to be observed when present, and a more accurate method of detection is needed. Therefore, p90<sup>rsk</sup> was immunoprecipitated from whole cell lysates using specific anti-p90<sup>rsk</sup> antibodies. Following immunoprecipitation, kinase activity was measured in the resulting immune complexes. The antibody anti-p90<sup>rsk</sup> was effective in bringing down a phosphotransferase enzyme in its active state (Fig. 5A). No activity was found if either the primary or the secondary antibodies were omitted from the assay. Similarly, no activity was found by immunoprecipitation of lysates from GM-CSF-treated cells using an irrelevant antibody (anti-GTPase-activating protein) instead of anti-p90<sup>rsk</sup>. The time course of activation by GM-CSF is shown in Fig. 5B. Immunoprecipitates from GM-CSF-treated cells contained an elevated p90<sup>rsk</sup> activity. Recovery of this elevated activity is dependent on the time of incubation with GM-CSF, peaking at 5–10 min. According to the anti-p90<sup>rsk</sup> immunoprecipitation results shown in Fig. 5 (A and B), activation due to GM-CSF is approximately 3.4-fold.

**In-gel Kinase Assay of Renatured p90<sup>rsk</sup>**—We next chose the in-gel kinase assay as a second approach to further analyze the behavior of p90<sup>rsk</sup> against GM-CSF cell stimulation. Under the conditions described for this assay (see "Experimental Procedures"), electrophoresed and subsequently renatured p90<sup>rsk</sup> (or p42<sup>MAPK</sup> in parallel experiments) from cell lysates was overlaid with [ $\gamma$ -<sup>32</sup>P]ATP. Because the phosphotransferase is locked at its corresponding relative mobility ( $M_r$ ) in the gel, it will produce a signal (evidenced by autoradiography) in that zone when incorporating radioactive ATP into the embedded substrate. As shown in Fig. 6A, 270 pM GM-CSF causes a rapid (5 min) increase in p90<sup>rsk</sup> and MAP phosphotransferase activities. When gels are analyzed by densitometry and [optical density  $\times$  band area] are plotted against time, an increase in p90<sup>rsk</sup> activation could be observed. This increment is still relatively low in comparison with MAP kinase activation (Fig. 6B).

**Role of p90<sup>rsk</sup> in GM-CSF Changes in Proliferating Cells**—An increase in p90<sup>rsk</sup> activation in response to GM-CSF was observed in mature cells. To investigate how this increment compares with a proliferating hematopoietic cell, we used a well established GM-CSF responsive cell, the erythroleukemia cell line TF-1. This cell line is dependent on the presence of cytokines (*i.e.* GM-CSF, interleukin-3, or erythropoietin) for its survival (37). As seen in Fig. 7A, GM-CSF increased thymidine incorporation in a dose-dependent manner. When p90<sup>rsk</sup> was analyzed in those cells (Fig. 7, B and C) we observed that: 1) When exponentially growing TF-1 cells were maintained in culture in the continuous presence of GM-CSF (cytokine-supplemented), they had a certain basal level of p90<sup>rsk</sup> phosphorylation and activity. 2) This basal level could not be significantly changed by an additional (0–15 min) exposure to GM-CSF after cell washing. 3) GM-CSF-starved cells (24 or 48 h) had no evidence of such basal level of phosphorylation and enzymatic activity. 4) When those GM-CSF-starved cells were challenged with GM-CSF *in vitro* for short (3 or 5 min) periods of time, the p90<sup>rsk</sup> activity quickly raised to a level higher than

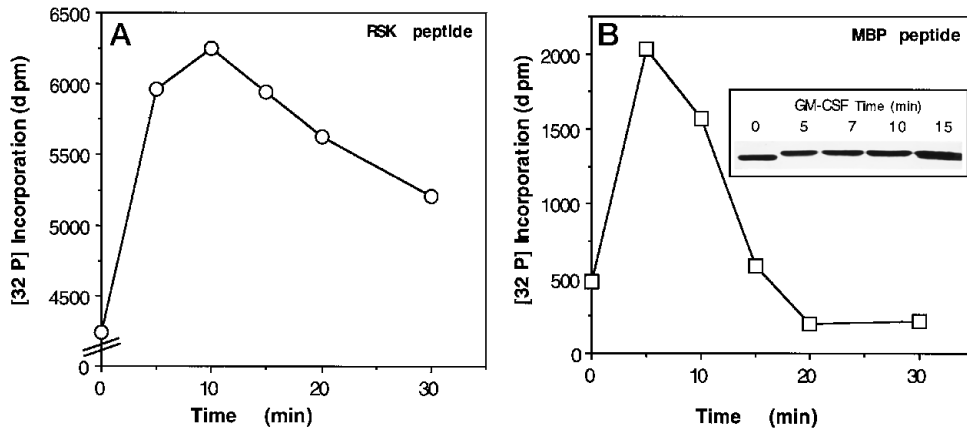


FIG. 3. Time course of p90<sup>rsk</sup> and p42<sup>MAPK</sup> activation by GM-CSF in whole cell lysates. Neutrophils were incubated for the indicated times at 37 °C with 270 pM GM-CSF. After this time, cells were lysed, and 20  $\mu$ l of whole cell lysates were used in a cell-free kinase assay with P81 phosphocellulose paper as described under "Experimental Procedures." The figure shows the <sup>32</sup>P incorporation into 50  $\mu$ M RSK peptide (A, p90<sup>rsk</sup> activity) and 500  $\mu$ M MBP peptide (B, p42<sup>MAPK</sup> activity). The inset represents a Western blot of cell lysates probed with anti-p42<sup>MAPK</sup> antibody.

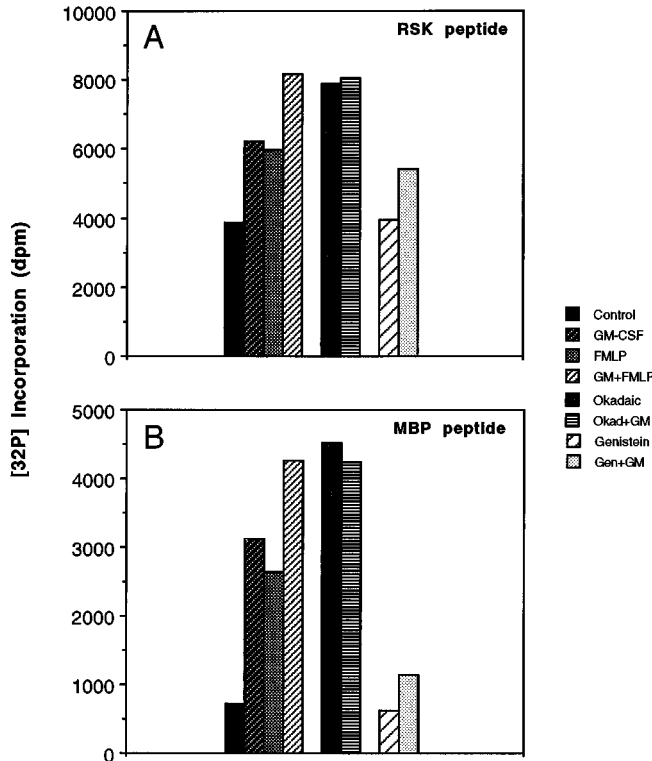


FIG. 4. Effect of neutrophil agonists and okadaic acid on p90<sup>rsk</sup> and p42<sup>MAPK</sup> enzymatic activities. Neutrophils were stimulated with 270 pM GM-CSF, 100 nM FMLP, 1  $\mu$ g/ml okadaic acid, 15  $\mu$ g/ml genistein, or a combination of these. Cell lysates were obtained, and 20  $\mu$ l of whole cell lysates were used in the kinase assay with phosphocellulose paper as described. The figure shows the <sup>32</sup>P incorporation into 50  $\mu$ M RSK peptide (A) and 500  $\mu$ M MBP peptide (B).

that observed in cytokine-supplemented cells, being increased 6.6-fold over controls. 5) Finally, these changes in p90<sup>rsk</sup> activity were paralleled, although at a slightly smaller quantitative level, by changes in MAP kinase, suggesting that starved TF-1 cells responded to GM-CSF by engaging in selected signal transduction in a fashion similar to that seen in mature neutrophils.

To further compare the response to GM-CSF in proliferative versus mature cells, we performed immunoprecipitation with specific anti-p90<sup>rsk</sup> antibodies. The cell substrates used were derived from neutrophils (as described above) and from two

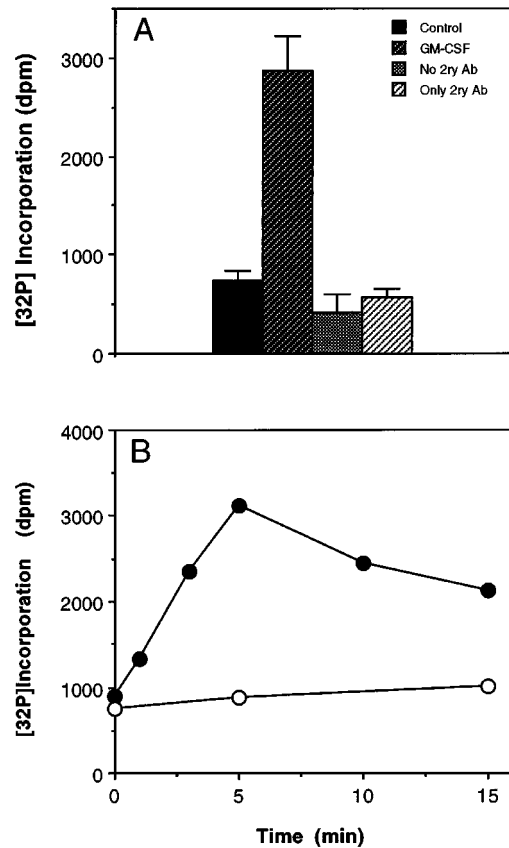
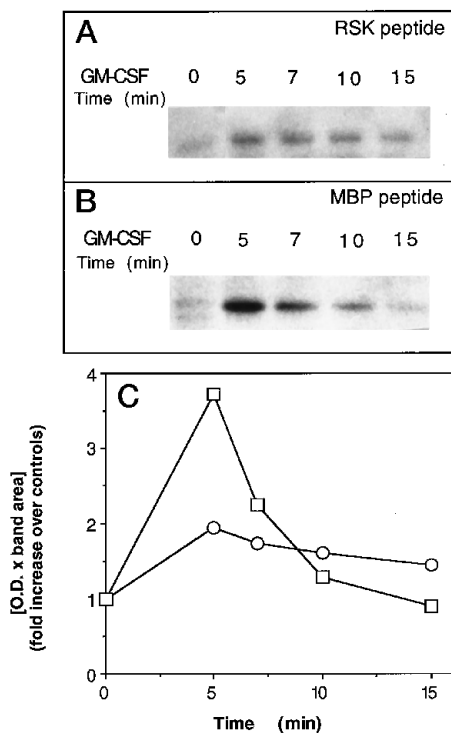


FIG. 5. p90<sup>rsk</sup> immune complex kinase assay. A, immunoprecipitation with anti-p90<sup>rsk</sup> antibodies was performed in total cell lysates from nonstimulated cells and cells stimulated with GM-CSF for 5 min. Immunoprecipitation controls included the absence of the secondary antibody (No 2ry Ab) or the primary antibody (Only 2ry Ab) during immunoprecipitation of lysates derived from GM-CSF-stimulated cells. The resulting immune complexes were used for the kinase assay using as substrates 50  $\mu$ M RSK peptide. Each point is the mean  $\pm$  S.E. of the values obtained in three separate experiments. B, neutrophils were treated with 270 pM GM-CSF (●) or left untreated (○) for the indicated period of time. Lysates corresponding to equal numbers of cells were immunoprecipitated with anti-p90<sup>rsk</sup> antibodies, and a kinase assay was performed in the immune complexes. The figure depicts the mean of four independent experiments.

proliferating cell lines: the TF-1 line and the nonhematopoietic cell line, HeLa (stimulated with 100 nM epidermal growth factor). When normalized to the same protein concentration,



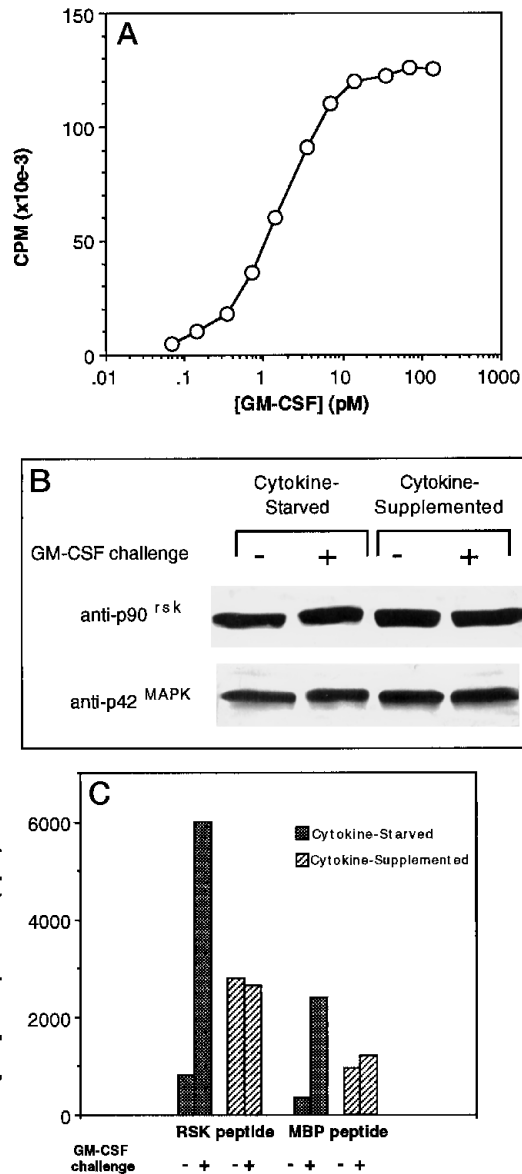
**FIG. 6. Time course of p90<sup>rsk</sup> and p42<sup>MAPK</sup> activation by GM-CSF in an in-gel kinase assay.** Neutrophils ( $2 \times 10^7$  cells/ml) were treated with 270 pM GM-CSF for the represented periods of time, and lysates were obtained as indicated. SDS gels were polymerized in the presence of 50  $\mu$ g/ml RSK peptide (A) or 250  $\mu$ g/ml MBP (B). The kinase reaction was performed in the renatured gels with [ $\gamma$ -<sup>32</sup>P]ATP as indicated under "Experimental Procedures." The figure shown is representative of three separate experiments. C, the films depicted in the upper panels were scanned at 633 nm in a laser densitometer (LKB Ultrascan, Pharmacia). From the x-ray film shown in A, the kinase of 90 kDa was scanned. From the x-ray film shown in B, the kinase of 42 kDa was scanned. Symbols represent: p90 kinase (p90<sup>rsk</sup>) (○) and p42 kinase (p42<sup>MAPK</sup>) (□). In the ordinate axes, the [optical density  $\times$  band area] values were normalized. One-fold activation represents [optical density  $\times$  band area] of kinases from unstimulated (control) cells, and the values of stimulated (GM-CSF) cells are referred to them.

the increments in enzymatic activity were 3.6-, 8.1-, and 10.2-fold, respectively.

#### DISCUSSION

We demonstrate here that p90<sup>rsk</sup> is present in the human neutrophil. Based on immunological and biochemical characteristics, the neutrophil p90<sup>rsk</sup> corresponds closely to the RSK kinase reported previously in various mammalian cell lineages (29). Specific phosphorylation of ribosomal S6 protein by S6Ks is a highly conserved response to the stimulation of quiescent cells by mitogens. The presence of these kinases in neutrophils was not totally expected, because these cells neither divide nor differentiate.

We further studied the presence of p90<sup>rsk</sup> and the response to GM-CSF in a type of hematopoietic proliferative cell, the erythroleukemia cell line TF-1. TF-1 cells are dependent on the presence of cytokines (*i.e.* GM-CSF, interleukin-3, or erythropoietin) for their survival. We found p90<sup>rsk</sup> was present in those cells. Further, exponentially growing TF-1 cells have a relatively high level of p90<sup>rsk</sup> (and MAP kinase) activation. However, this level nearly disappears when cells are deprived of the cytokine only to rapidly increase again when they are further exposed to GM-CSF. Because TF-1 cells that are cytokine-deprived do not proliferate (37), short challenge by GM-CSF might force them back from G<sub>0</sub> to the cell cycle. As shown here, this exit/re-entry and concomitant cell proliferation might re-



**FIG. 7. p90<sup>rsk</sup> and MAP kinases in response to GM-CSF in erythroleukemia TF-1 cells.** A, [<sup>3</sup>H]thymidine uptake in TF-1 cells in response to GM-CSF stimulation. Starved TF-1 cells were washed once in RPMI 1640 containing 2% FCS and plated in 96-well plates at  $2 \times 10^4$  cells/well in complete medium with or without cytokine, incubated for 48 h before a 6-h pulse with [<sup>3</sup>H]thymidine (0.5  $\mu$ Ci/well). The cells were then harvested, and radioactivity was counted. B, effect of GM-CSF on the relative mobility of p90<sup>rsk</sup> and MAP kinase p42. Equal number of either exponentially growing, GM-CSF-supplemented (*Cytokine-Supplemented*) or 48-h GM-CSF-starved (*Cytokine-Starved*) and alive (by trypan blue exclusion) TF-1 cells ( $5 \times 10^6$ /ml) were stimulated with 270 pM GM-CSF for 5 min. Cell lysates were obtained, and Western blots were probed with anti-p90<sup>rsk</sup> or anti-p42<sup>MAPK</sup> antibodies. C, measurement of p90<sup>rsk</sup> and p42<sup>MAPK</sup> enzymatic activity. Lysates obtained as indicated for B were used to measure the phosphotransferase activity of both kinases as indicated under "Experimental Procedures." Data are representative of three separate experiments with essentially similar results.

quire the participation of p90<sup>rsk</sup> and/or MAP kinase. Because there seems to be less difference in GM-CSF-induced MAP kinase activity than there is with p90<sup>rsk</sup> (Fig. 7), RSK could be more definitely involved in cell cycling.

The GM-CSF-induced p90<sup>rsk</sup> increment in enzymatic activity in neutrophils was quantitatively smaller than that observed in starved TF-1 cells challenged with GM-CSF or in HeLa cells activated with epidermal growth factor. Thus, p90<sup>rsk</sup>, although

present in mature hemopoietic cells, is activated at an extent lower than in the proliferative counterpart. Whether this is an effect or a contributing cause of transcriptional paucity in cells that have reached maturity remains to be clearly elucidated. Different sets of substrates might be targeted in proliferative and mature cells. For instance, MAP kinase in proliferative cells translocates to the nucleus and targets transcription factors. In mature cells, MAP kinase targets membrane enzymes like cytosolic phospholipase A<sub>2</sub> whose activation is more in line with the functionality of phagocytes (*e.g.*, release of lipidic mediators of inflammation).

What substrates are used by p90<sup>rsk</sup> to transduce signals downstream is a subject of intense study in different labs. To date, only two kind of substrates have been found for p90<sup>rsk</sup>: transcription factors and the G subunit of phosphatase 1 that regulates the activation of glycogen synthase (44). As for mature neutrophils, we can only theorize that p90<sup>rsk</sup> might regulate the expression of *c-fos*. *c-fos*-specific transcripts are constitutively expressed in high levels in neutrophils (45) that are further increased in presence of GM-CSF (46).

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