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Ribosomal p70S6K Basal Activity Increases upon Induction of Differentiation of Myelomonocytic Leukemic Cell Lines HL60, AML14 and MPD

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Abstract

The role of ribosomal p70S6K in the cell cycle has been studied extensively, and it is known that this enzyme is crucial for cell advancement through G₁. Conversely, the participation of p70S6K in cell differentiation is not well understood. We have studied the response of p70S6K to the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) in three differentiation-capable leukemic cell lines (MPD, AML-14 and HL-60) and in normal mature neutrophils. Immature leukemic cells starved for 16 hours showed a robust (~3.5-fold over controls) p70S6K phosphorylation on T⁴²¹/S⁴²⁴ residues in response to an acute (5 min) 10 nM GM-CSF stimulation. On the other hand, cells that had been induced to differentiate and express granulocytic phenotypes, showed an increased (~6-fold) basal level of p70S6K T⁴²¹/S⁴²⁴ phosphorylation over immature cells, as well as an increased baseline tyrosyl phosphorylation of the GM-CSF receptor β subunit (GM-CSF•R β). However, these cells displayed a weak (~1.4-fold over controls) response to GM-CSF even at prolonged incubation times (20 min). *In vitro* p70S6K enzymatic activity paralleled p70S6K T⁴²¹/S⁴²⁴ phosphorylation in both high basal, unstimulated, levels in immature cells and a low degree of response to GM-CSF. Lastly, peripheral blood mature neutrophils had low basal GM-CSF•R β and p70S6K activity, with both parameters being robustly stimulated following addition of GM-CSF, a situation in contrast with the cell lines, indicative perhaps of their incomplete terminal differentiation. In summary, these findings show the increase in basal phosphorylation of p70S6K upon granulocytic differentiation of myeloid leukemic cells and their responses to GM-CSF that are closely paralleled with tyrosyl phosphorylation of its receptor, and help in pointing to specific cell signaling molecules that are different in leukemic blasts from normal leukocytes.

Keywords

ribosomal p70S6K; leukemic cell lines; AML; MPD; GM-CSF

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1. INTRODUCTION

Hematopoiesis is an advantageous system for the study of cell differentiation. Bone marrow stem and progenitor cells commit themselves to discrete lineages that give rise to the different types of mature circulating blood cells. Acute leukemia results when the process of differentiation is arrested. The specific mechanisms leading to maturation arrest in leukemia are complex and not completely understood, although abnormalities of the phosphorylation/dephosphorylation of molecular links in signal transduction pathways have been implicated in some cases. The process of signal transduction that links the cell membrane with nuclear gene expression is currently under intense study in both normal and malignant cell systems.

The p70S6K enzyme plays a role in the control of translation of mRNAs that possess a polypyrimidine tract at their 5' ends [1] and in protein synthesis [2], as it catalyzes the phosphorylation of the S6 protein in the 40S subunit of eukaryotic ribosomes [3-5]. p70S6K has been shown to be necessary for progression through the cell cycle, specifically for the G₁ → S transition [6]. S6Ks are in actuality two kinases, S6K1 and S6K2, products of two different genes. Both enzymes phosphorylate ribosomal S6 protein and both are highly homologous, but they differ in their N- and C- terminal regulatory domains. The S6K1 isoforms are termed p70S6K-α2 ('p70') and p85S6K-α1 ('p85'); the latter a longer form with nuclear localization [7,8]. The S6K2 isoforms are termed p70S6K-β2 (with cytoplasmic and nuclear localization) and p70S6K-β1 (with nuclear localization) [9-12]. Ribosomal S6K1 has multiple phosphorylation sites [13] and its activation is inhibited by rapamycin [14-16]. The role of ribosomal p70S6K in cell cycle has been well-established, but there is little information regarding a role for p70S6K in cell differentiation.

We demonstrate here that in differentiation-capable leukemic cell lines (especially MPD), p70S6K is activated by GM-CSF. However, when these cells are induced to mature, the basal level of the receptor (GM-CSF•R) phosphorylation as well as p70S6K phosphorylation and enzymatic activity increase substantially and can be only weakly enhanced by activation with GM-CSF.

2. MATERIALS AND METHODS

2.1. Reagents and antibodies

The promyelocytic leukemic (M2 myeloblast-derived) HL-60 cell line was from The American Type Culture Collection (Rockville, MD); AML-14, 3D10 and MPD were established by the authors [17,18]. Tissue culture material was from Becton Dickinson (Oxnard, CA) and Costar (Cambridge, MA); GM-CSF was from Sandoz (East Hanover, NJ); G-CSF was from Amgen (Thousand Oaks, CA); PMA, PKA inhibitor, calphostin, anti-mouse IgG (agarose beads), differentiation kit, trypan blue, NBT reduction kit and myelin basic protein were from Sigma (St. Louis, MO); electrophoresis chemicals were from Bio-Rad Laboratories (Richmond, CA); [γ -³²P]ATP (30 Ci/mmol) was from Amersham Pharmacia Biotech (Arlington Heights, IL); ion-exchange chromatography cellulose phosphate paper was from Whatman (Hillsboro, OR); anti-p70S6 kinase (H-9) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-PY (PY20 clone), was from UBI (Lake Placid, NY); anti-GM-CSF•R, common β (CDw131) chain, was from BD Biosciences (Franklin Lakes, NJ); p70S6 kinase peptide substrate KKRNRRLTK and anti-phospho(T⁴²¹/S⁴²⁴)-p70S6 kinase antibodies (polyclonal) were from Cell Signaling (Beverly, MA).

2.2. Cells

Neutrophils. Peripheral blood neutrophils were isolated based on a protocol described by English and Andersen [19]. Blood (50-55 ml) was collected from the antecubital vein of healthy individuals (who signed an Institutional Review Board-approved consent form)

using sodium citrate as anticoagulant and neutrophils were separated in a Ficoll-Histopaque discontinuous gradient. Viability is usually $>98 \pm 2\%$ as per trypan blue exclusion. Cells were resuspended in RPMI at the concentration of 2×10^6 cells/ml. Leukemic cells. HL-60, AML14 and its differentiated subline AML14.3D10, and pluripotent MPD cells were grown at 37°C in a $5\% \text{CO}_2$ incubator in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine and 5 mg/ml gentamicin. Cell density was maintained between $0.1\text{--}1.0 \times 10^6/\text{ml}$. HL-60 cells can be induced to differentiate into granulocyte-like cells by incubation with dimethyl sulfoxide (DMSO) [20-22]. Once they reach the neutrophilic phenotype, these cells lose their capability to proliferate and start responding as mature neutrophils. The AML-14 cell line is a blast line, from which the eosinophil-committed AML-14.3D10 subclone was isolated following long-term incubation of AML14 with GM-CSF, IL-3 and IL-5, which share a receptor subunit and activate similar signal transduction pathways [17,23]. AML-14.3D10 can be induced to “switch” lineage commitment from eosinophil to neutrophil following exposure to all-trans retinoic acid (ATRA) [24]. HL-60 cells were induced to differentiate by incubation with 1.25% (vol/vol) DMSO for 3 days in order to achieve the neutrophilic phenotype (dHL-60). To induce differentiation of the MPD cell line to mature neutrophils (gMPD), cells were grown at 37°C in a $5\% \text{CO}_2$ incubator in RPMI 1640 medium continuously supplemented with 20 pM granulocyte colony-stimulating factor (G-CSF) for 5 days. In several experiments, starved or quiescent AML14, HL-60 and MPD cell populations were prepared by depriving them of serum in the last 16 hours of culture prior to the experiment and challenge with GM-CSF. Viability assays were routinely conducted with 0.4% trypan blue stain in cell preparations prior to all analyses.

2.3. Assessment of cell differentiation

Following 80 hours of DMSO incubation, about 75% of HL-60 cells had matured to at least the myelocytic stage and had acquired the ability to release superoxide anion in response to bacterial extracts challenge. To ascertain that DMSO produced the desired neutrophilic phenotype in HL-60 cells, cells were assayed for capacity to reduce nitroblue tetrazolium (NBT) following treatment with non-viable bacterial extracts. Maturing cells contained formazan deposits as dark, irregularly-shaped crystal inclusions in the cytoplasm. Additionally, flow cytometric analysis of surface expression of differentiation-related antigens was performed with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against CD11b, CD11c, CD13, CD14, CD16 or CD54 [25].

2.4. Immunoprecipitation and Western blotting analyses

The procedure was based on our previous report [26] with some modifications, as follows. Cells were resuspended in RPMI at 3×10^6 cells/ml density and were 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 boiling SDS solution (1% SDS in 10 mM Tris-HCl, $\text{pH } 7.4$). Samples were boiled in a heat block for 10 min with frequent vortexing to achieve complete dissolution, taken to an ice bucket and mixed with 0.3 ml cold ddH_2O and 0.4 ml cold, Triton X-100-based, lysis buffer (12 mM Tris-HCl, $\text{pH } 7.2$, 0.75 mM NaCl, 100 mM sodium orthovanadate, 10 mM phenylmethylsulfonyl fluoride, 0.2 mM β -glycerophosphate, $5 \text{ }\mu\text{g/ml}$ each of aprotinin, pepstatin A and leupeptin, and 0.12% Triton X-100). The resulting lysates were mixed with the primary $2 \text{ }\mu\text{g/ml}$ antibody (anti-p70S6K) and anti-rabbit (IgG, whole molecule) antibody conjugated to agarose beads in lysis buffer for 4 hours at 4°C . 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, $\text{pH } 7.4$, 400 mM LiCl) and twice with buffer B (10 mM Tris-HCl, $\text{pH } 7.4$, 100 mM NaCl, 1 mM EDTA). Immune complex beads were resuspended in a final volume of $60 \text{ }\mu\text{l}$ with lysis buffer and mixed with 2X-SDS sample buffer ($1:1 \text{ v/v}$) for subsequent protein gel

electrophoresis/immunoblotting. Resulting gels were transferred onto PVDF membranes and used for immunoblotting with anti-phospho (T^{421}/S^{421}) p70S6K antibodies. In several experiments, parallel blots were probed with the same antibody used for immunoprecipitation (anti-p70S6K), to confirm that protein loading was similar (kept at <5%). Densitometry was performed with the Un-scan-it Gel software (Silk Scientific, Inc., Orem, UT).

2.5. Immunocomplex p70S6 kinase assay

Ribosomal p70S6K enzymatic activity was quantified by using an immunocomplex kinase assay as reported previously, tailored to measure this particular kinase activity in human neutrophils [27]. Cells were resuspended in RPMI at 3×10^6 cells/ml density and were 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 ice-cold, TRIS-based, lysis buffer (see above for composition) and incubated on ice for 15 minutes with occasional vortexing. After centrifugation (7,000 $\times$ g, 1 min, 4 °C), supernatants were mixed with the antibody conjugated to agarose beads as indicated above. Immune complex beads were resuspended in a final volume of 40 μ l with 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 μ M of the S6 kinase substrate peptide KKRNRSLTK in freshly-prepared kinase buffer (13.4 mM HEPES, pH 7.3, 25 μ M $MgCl_2$, 30 μ M Na_2VO_3 , 5 mM *p*-nitrophenyl phosphate, 2 mM EGTA, 2 mM cAMP-dependent kinase inhibitor TTYADFIASGRTGRRNAIHD, 0.420 μ Ci [γ - ^{32}P]ATP (7 nM), and 68 μ M unlabeled ATP). To initiate the phosphotransferase reaction, aliquots (20 μ l) 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 30 °C for 20 min and terminated by blotting 40 μ l of the reaction mixture onto P81 ion exchange chromatography cellulose phosphate papers.

Statistical Analysis

Data are presented as the mean $\pm$ SEM. The difference between means was assessed by the Single Factor Analysis of Variance (ANOVA) test. Probability of less than 0.05 ($p < 0.05$) or less than 0.001 ($p < 0.001$) was considered to indicate a significant difference.

3. RESULTS

Quiescent MPD (serum starved for 16 hours) cells incubated with GM-CSF for short periods of time displayed a robust increase in p70S6K phosphorylation of key residues T^{421}/S^{421} (Fig. 1A, left group of bands). This started at 5 min of GM-CSF stimulation and continued up 20 min in the experiments shown. However, when gMPD cells were induced to differentiate to neutrophils (labeled as “gMPD” in the figure) prior to GM-CSF challenge, two major changes occurred. First, the gMPD cells responded only weakly to GM-CSF (Fig. 1A, middle group of bands). Densitometric analysis of the p70S6K- T^{421}/S^{421} phosphorylated bands, taking into account protein loading (Fig. 1B), indicated an increase of ~1.4-fold over controls in gMPD cells. This was in contrast with an increase of ~3.5-fold over controls in the starved MPD cells alluded to above. Second, the basal level of p70S6K phosphorylation (i.e., cells not stimulated with GM-CSF) was greatly (~6-fold) elevated in gMPD over that of MPD cells (Fig. 1A, zero time). This is seen in phospho-bands in immunoblots as both an increase in the darkness of the signal to the antibody and as an upward mobility shift in the bands, consistent with an increase in the phospho content of the p70S6K molecule [28]. These heavily phosphorylated bands have the same relative mobility (M_r) as the positive controls for these experiments, serum-stimulated fibroblasts 3T3 cells (Fig. 1A, right group of bands). The elevated baseline level of MPD vs. gMPD is clearly demonstrated in Fig. 1C in which quantified phosphorylation is plotted for both cell lines in the absence of GM-CSF.

We next measured p70S6K enzymatic activity *in vitro* (Fig. 2). As above, immature cells (MPD) were “quiescent” i.e., serum starved for 16 hours. Incubation of cells with GM-CSF produced a time-dependent increase in activity that paralleled the data presented in Fig. 1 for T⁴²¹/S⁴²¹ phosphorylation. Seen again was a high level of basal activity in gMPD cells (1,533±150 dpm) as compared to that of MPD cells (502±30 dpm), and a weak, but significant, response to GM-CSF on the former (maximum response at 5 min increased ~1.36-fold; compared ~3-fold for MPD cells during their maximum response at 10 min). To ascertain if the results obtained with the MPD cell line would be similar to other cell lines, we performed experiments with the line AML14 and its eosinophilic, more mature, subclone, AML14.3D10. Fig. 3A,B shows that AML14.3D10 has a higher p70S6K phosphorylation level at baseline than AML-14 for equal amounts of protein in the gels (anti-p70S6K panels). Similarly, differentiated HL-60 (dHL-60) has a higher background than undifferentiated HL60. This is paralleled by p70S6K enzymatic activity on those cells (Fig. 3C). All this serves to indicate that the results observed are not unique for just one single cell line, but are extrapolatable to at least another 2 commonly -studied leukemic cell lines.

We next examined the activation status of the GM-CSF receptor (GM-CSF•R) in immature vs. differentiation-induced leukemic cell lines, to ascertain if the results presented above could be explained by changes in early events in cell signaling. We performed immunoprecipitation with anti-GM-CSF•R-beta subunit (GM-CSF•R_β) antibodies. The immunocomplexes were further analyzed by immunoblotting using anti-phosphotyrosine (PY) antibodies. This is a valid indicator of receptor activation, since it is known that after binding of GM-CSF to the α/β receptor complex, the intracellular tail of the β subunit is phosphorylated on tyrosine [29]. Fig. 4A shows that the basal level of tyrosyl phosphorylation of GM-CSF•R_β in MPD cells is negligible. However, upon stimulation with GM-CSF, strong phosphotyrosine activation is seen over the time course. Contrary to this, Fig. 4B shows an increase in basal phosphorylation in neutrophilic MPD (gMPD) in the absence of GM-CSF, that can only marginally be elevated after 3 min of GM-CSF stimulation, a situation paralleling the p70S6K results observed in Figs. 1 and 2. Protein loading is equivalent as shown in the bottom panel of Fig. 4B and densitometry of the receptor band is presented in Fig. 4C. Similar results were obtained with AML-14.3D10 and with dHL-60 (not shown). Thus, the events observed in p70S6K for the two different stages of maturation of leukemic cells correlate with those seen with the GM-CSF receptor.

The final series of experiments analyzed the level of p70S6K in normal neutrophils isolated from peripheral blood. These cells were considered here as the fully mature, physiological counterpart of leukemic cells induced to differentiate. The neutrophils had a very low background of p70S6K phosphorylation and enzymatic activity (Fig. 5). Addition of GM-CSF induced a robust response in S6K phosphorylation and activity.

4. DISCUSSION

We have shown that GM-CSF induces a robust activation of p70S6K in starved, immature leukemic cells that is reduced following maturation induction. Further, we have demonstrated a high parallelism between GM-CSF•R_β tyrosyl phosphorylation and p70S6K activity. Both basal activities increase with induction of differentiation of leukemic cell lines. We made use of three cell lines that can be induced to express a more mature phenotype (HL-60, AML-14 and MPD) [17,18]. The significant activation of p70S6K that we have described here in undifferentiated leukemic cell lines upon exposure to GM-CSF, could be explained considering the important role of this enzyme in facilitating protein synthesis. The immature leukemic cells used in the experiments described here were serum-starved for 16 hours. It is known that in those conditions, cells become arrested in the G₀

phase of the cell cycle and can be induced to exit their arrest upon exposure of cell cultures to cytokines, as was done here. However, we expected that the maturation-induced cell lines, which are not proliferative, would have less basal p70S6K activity.

What we observed was an increased basal level of p70S6K phosphorylation and enzymatic activity and GM-CSF•R β phosphorylation in the more mature populations that was not seen in neutrophils. Three possible explanations can be offered for this divergence: (a) the signaling pathways directing terminal differentiation of normal neutrophils and the leukemic cell lines differ, leading to the observed disparate activities; (b) the leukemic cells might harbor a mutated kinase leading to increased baseline phosphorylation, or might be deficient in a phosphatase that would ordinarily downregulate p70S6K and/or the GM-CSF receptor; and (c) there is an incomplete terminal differentiation of the cell lines, as produced by standard induction with DMSO or cytokines (like G-CSF), rather than a fundamental difference between the biology of malignant and normal cells. The latter seems to be the more straight forward explanation.

Although it has been suggested that p70S6K may play a role in the differentiation of some cell types, such as myocytes [30-33], there are few prior studies of p70S6K activity in hematopoietic cells. A role for p70S6K in G-CSF-induced neutrophilic differentiation has been suggested [34]. The same authors showed that wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K), partially inhibited G-CSF-induced p70S6K activity, with p70S6 kinase being hyperphosphorylated by G-CSF in transferrin receptor (Trf-R) positive (+) cells, but not in Trf-R(-) cells [35]. G-CSF is able to increase the functional maturation of HL-60 cells treated with DMSO and, concomitantly, induces activation of p70S6K and nuclear translocation of activated STAT3, but not activation of MAPK [36]. Interestingly, GM-CSF acted as a priming agent to make G-CSF responsive to MAPK [36].

The role of this insulin receptor substrate-1 (IRS-1) has been recently shown to coincide with the granulocytic differentiation of haematopoietic cell lines [37]. The 32D IGF-IR/IRS-1 cell line, that has a combined expression of the insulin-like growth factor (IGF) receptor and its substrate, is a proliferating, transformed cell that, following serum starvation, responded robustly to IGF in terms of p70S6K phosphorylation. This results are in accordance with the data presented here for MPD. The relationship between the p70S6K signal pathway and the interleukin-6 production was reported in both normal and leukaemia myeloid cells [38]. Although we have not studied the role of this key cytokine in the biological process of differentiation induced, our results agree with those of Birkenkamp *et al.* in that they have found that mature cells (peripheral blood monocytes) do behave differently than leukemic AML blasts. Rapamycin inhibits cell proliferation on the latter but actually activated IL-6 secretion in monocytes. Lastly, the role of c-myc in the downstream events of p70S6K during granulocytic differentiation of myeloid leukaemia cells has been recently reported [39]. In that study, Kanayasu-Toyoda *et al.* show in Fig. 2 that Trf-R+ (proliferating) cells respond to G-CSF in terms of p70S6K phosphorylation. Interestingly, Trf-R- (differentiating) cells also respond to G-CSF but at a lower extent. Our results of gMPD having lower phosphorylation and activity versus MPD are in accordance with those previously reported results.

Our observations of the divergent p70S6K phosphorylation and activity, as well as the status of the GM-CSF•R in mature neutrophils vs. differentiated leukemic cell lines, provides an opportunity for further understanding leukemic transformation and the completeness of differentiation induction. Further information on divergent functionality of signaling molecules could ultimately result in targeted strategies for improved clinical treatments.

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ABBREVIATIONS

GM-CSF	granulocyte-macrophage colony-stimulating factor
G-CSF	granulocyte colony-stimulating factor
p70S6K	ribosomal p70 S6 kinase
HL-60	human promyelocytic leukemia
dHL-60	differentiated HL-60
AML	acute myelocytic leukemia
AML3D10	eosinophilic AML
MPD	myeloproliferative disorder cell line
gMPD	granulocytic MPD
GM-CSF•Rβ	common β (CDw131) subunit of the GM-CSF cell membrane receptor

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Figure 1. Phosphorylation of p70S6K in undifferentiated (MPD) and differentiated (gMPD) cells (A) Quiescent MPD cells (○) or differentiated MPD (gMPD) (●) cells were stimulated with 10 nM GM-CSF for the indicated lengths of time (in minutes) 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(T⁴²¹/S⁴²¹)-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) Densitometry analysis of p70S6K phosphorylation. Densitometry of 70-kDa bands in experiments with results similar to that shown in (A) were scanned and the parameter [density × Area] was quantified for the phospho blot and for the protein blot. Then, the ratio Phospho/Mass was calculated. Shown is the mean ± SE of three independent experiments. (C) Same as (B) but this time referring only to the 70-kDa in the absence of GM-CSF. Responses to GM-CSF of MPD cells were significantly (**, $p < 0.001$) higher than control (time zero) at every time point, and responses in gMPD cells were significantly (*, $p < 0.05$) higher than control at 10 minutes.

Figure 2. Effect of GM-CSF on p70S6K enzymatic activity in MPD cells

Cell populations at approximately the same density: $1.1\text{--}1.6 \times 10^6$ cells/ml for quiescent MPD (○) and $1.0\text{--}1.2 \times 10^7$ cells/ml for differentiated, gMPD (●), were incubated with GM-CSF for the indicated times. Cells lysates were prepared and protein measured (by Bradford), and adjusted for all samples as to contain similar mg/ml concentrations. Then, samples were subjected to immunoprecipitation with specific anti-p70S6K antibodies, followed by an *in vitro* kinase activity assay in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $150\text{ }\mu\text{M}$ of KKRNRTLTK (p70S6K peptide substrate that bears the phosphorylation site of natural ribosomal S6 protein). Results are the mean $\pm$ SE ($n=4$ for MPD and $n=3$ for gMPD) performed in duplicate. Changes over controls that were significant are indicated as **, $p<0.001$ and *, $p<0.05$.

Figure 3. Comparison of p70S6K phosphorylation and enzymatic activity in two other immature and mature leukemic cell lines

(A) Aliquots of the eosinophilic cell line AML-14 and its mature counterpart, AML-14.3D10 (labeled as “3D10” in the figure), and the promyelomonocytic cell line HL-60 and its mature counterpart, (labeled as “dHL-60” in the figure) were lysed in boiling SDS buffer, followed by immunoprecipitation (I.P.) with anti-p70S6K and analyzed by Western blotting (W.B.) with anti-phospho(T⁴²¹/S⁴²⁴)-p70S6K antibodies, as well as with anti-p70S6K antibodies, after stripping the former one, to confirm equal protein loading. (B) Densitometry of 70-kDa bands in experiments with results similar to that shown in (A). (C) Enzymatic activity of p70S6K, assayed as indicated in legend of the previous figure. Results are the mean $\pm$ SE performed in duplicate. All mature cells showed basal phosphorylation backgrounds that were statistically significantly (**, $p < 0.001$ and *, $p < 0.05$) higher than those found in immature cells.

Figure 4. Analysis of the GM-CSF receptor β subunit

Starved MPD cells (**A**) or mature MPD (gMPD) (**B**), were challenged with 1 nM GM-CSF for the indicated lengths of time (in min) and cell lysates were prepared.

Immunoprecipitations with anti-GM-CSF receptor common β -subunit antibodies yielded immunocomplex beads that were electrophoresed and proteins were transferred to PVDF membranes. The resulting blots were subjected to Western blotting with anti-phosphotyrosine (PY) or anti- β -subunit antibodies. The figure depicts the region between 116-170 kDa. (**C**) Ratio PY/receptor β -subunit mass. The 130-kDa β subunit band from the previous blots were scanned and the amount [Density $\times$ Area] was quantified from the PY-blot and for the β -subunit (protein) blot. Then, the ratio PY/Mass was calculated. Empty bars = MPD cells; gray bars = differentiated gMPD cells. Shown is a representative experiment out of three with similar results. Statistical analysis as indicated in the legend of previous figures.

Figure 5. Effect of GM-CSF on neutrophil p70S6K activity and phosphorylation

Freshly isolated neutrophils from peripheral blood, resuspended in RPMI at the concentration of 5×10^6 cells/ml, were incubated with GM-CSF for the indicated lengths of time. Cells lysates were subjected to immunoprecipitation with anti-p70S6K and analyzed for T⁴²¹/S⁴²⁴ p70S6K phosphorylation in Western blots. 70 kDa bands were analyzed by densitometry and the results are indicated in bars. Samples treated similarly and processed in parallel were assayed for *in vitro* enzymatic activity (○).