Granulocyte-macrophage colony-stimulating factor stimulation results in phosphorylation of cAMP response element-binding protein through activation of pp90RSK

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Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) promotes the proliferation and maturation of myeloid progenitor cells in vitro and in vivo. The GM-CSF receptor consists of an alpha and a beta subunit, both of which are members of the hematopoietin receptor superfamily. The alpha subunit binds ligand and plays a critical role in activating the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway. The beta subunit does not bind ligand, but is essential for signal transduction. The alpha and beta subunits of the GM-CSF receptor form a heterodimeric complex, which lacks a tyrosine kinase motif or GTP-binding site. The association of GM-CSF with its receptor mediates a variety of biologic responses in myeloid cells, including proliferation, maturation, and enhanced effector cell function.

Signals transduced by the GM-CSF receptor result in the phosphorylation of several kinases, including ERK, Ras, Raf, and JAK 2. Activation of these kinases results in rapid and transient induction of immediate early genes independently of protein synthesis, including early growth response gene-1 (egr-1) and c-fos. The egr-1 gene encodes a zinc finger containing DNA-binding protein that can act as a positive or negative regulator of cell growth. egr-1 has been implicated in the regulation of B lymphocyte development and proliferation. Antisense egr-1 blocks differentiation of granulocytes but not monocytes. We previously demonstrated that egr-1 gene regulation in response to GM-CSF requires a cyclic adenosine monophosphate (cAMP) response element (CRE) within the −116 nucleotide region of the human egr-1 promoter. Phosphorylation at serine 133 was shown to activate the CRE-binding protein, CREB, through a protein kinase A-independent pathway, so we intended to determine which kinase is responsible for GM-CSF-mediated CREB activation.

Recently, the serine/threonine kinase, pp90RSK (ribosomal S6 kinase), was shown to be activated in response to GM-CSF in the human myeloid leukemic cell line, TF-1. RSK is a member of a family of kinases that contains 2 highly conserved catalytic domains. RSKs are thought to regulate the transition of cells from G0 to G1, in part by the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) kinase (MEK) signaling pathway. GM-CSF may also induce multiple cell survival signals that involve phosphorylation of BAD (Bcl-2/Bcl-XL–associated death promoter) through a MEK-dependent pathway. In humans, abnormalities of RSK2 activation by the MEK pathway have been associated with X-linked mental retardation occurring in Coffin-Lowry syndrome. The precise role of pp90RSK and its downstream targets during myeloid proliferation has not been defined.
Several reports have suggested that pp90RSK activation results in phosphorylation of the transcription factor, CREB. Treatment of PC12 cells with nerve growth factor (NGF), epidermal growth factor (EGF), or 12-O-tetradecanoyl phorbol-13-acetate (TPA) results in CREB phosphorylation. Further studies demonstrated that RSK2 phosphorylates CREB on serine 133 after NGF treatment of PC12 cells. In human melanocytes, RSK2 is the kinase that activates CREB in response to serum and fibroblast growth factor. From these studies, we hypothesize that pp90RSK is the kinase that phosphorylates CREB at serine 133 in TF-1 cells. Here we report that GM-CSF stimulation of TF-1 cells results in pp90RSK activation. Immune complex kinase assays revealed that pp90RSK phosphorylates CREB in response to GM-CSF. Our data suggest that activation of pp90RSK is critical for the transcriptional activation of egr-1 in cells treated with GM-CSF. From these results, we conclude that activation of pp90RSK leads to egr-1 transcription and CREB phosphorylation through a MEK-dependent pathway.

Methods

Cells and reagents

TF-1 cells (ATCC; Rockville, MD), a human GM-CSF–dependent myeloid leukemic cell line, were cultured in RPMI medium containing 10% fetal calf serum (FCS; HYCLONE; Logan, UT), L-glutamine (2 mmol/L; Sigma; St Louis, MO), gentamicin (0.01 mg/mL; Sigma), and penicillin (100 U/mL); streptomycin (100 mg/mL) at a ratio of 1 U/mL to 1 mg/mL (Sigma). Cells were cultured in nonadherent tissue culture plates (100 × 15 mm) at 37°C and 1% CO2. TF-1 cells were stimulated with rhGM-CSF (1 nmol/L) or TPA (50 ng/mL). TF-1 cells were harvested after 4 hours of stimulation for 10 minutes served as the negative control, and TPA stimulation for 10 minutes as the positive control. Cells were lysed in immune complex kinase (ICK) lysis buffer (10 mmol/L Tris, pH 7.6, 50 mmol/L NaCl, 1% Triton X-100, 30 mmol/L sodium pyrophosphate, 0.1 mmol/L sodium molybdate, 50 mmol/L NaF, 1 mmol/L EGTA, 1 mmol/L sodium vanadate, 5 mmol/L benzamidine, 1% aprotinin, and 1 mmol/L PMSF) and sonicated for 5 seconds. Total soluble protein (600 µg) was incubated with 2 µg of anti-RSK1 antibody (Santa Cruz; specific for pp90RSK1) for 1 to 2 hours at 4°C and precipitated by overnight incubation with protein A/G agarose beads (Santa Cruz). Affinity-purified GST-CREB fusion protein (11 µg; gift from G. Perini) was used as substrate. The fusion protein was isolated by using previously determined conditions. The GST moiety was removed by cleavage, demonstrating phosphorylation of CREB and not GST, thereby indicating that the CREB moiety is phosphorylated. The reaction mixtures totaled 30 µL, which contained 600 µg of immunoprecipitated cell extract (as described above), 30 mmol/L HEPES, pH 8.0, 10 mmol/L MgCl2, 1 mmol/L DTT, 20 µmol/L ATP, 5 mmol/L benzamidine, and .148 MBq P gamma-ATP. Reaction mixtures were incubated for 30 minutes at 30°C. SDS-sample buffer was added to stop reactions. Samples were loaded and electrophoresed on a 10% SDS-polyacrylamide gel, followed by Coomassie blue dye staining. The gel was destained, dried, and autoradiographed. The bands that were detected on the autoradiogram represented phosphorylated GST-CREB. These bands were excised and quantitated by scintillation counting.

Western blotting

Nuclear and cytoplasmic extracts from TF-1 cells were isolated as previously described. Twenty micrograms of protein in nuclear and cytoplasmic fractions from diluent- or GM-CSF–treated cells were electrophoresed on a 10% SDS-polyacrylamide gel. The blot was probed with 3 µg/mL of RSK antibody (UBI; Lake Placid, NY). For the Western blot time course, TF-1 cells were serum- and factor-starved, and placed in RPMI containing 0.5% bovine serum albumin (BSA) for 24 hours. In the presence of serum, basal activity levels are generally high. To minimize basal activity, we performed experiments in the absence of serum and in 0.5% BSA as previously described. Cells were treated with GM-CSF (1 mmol/L) for 1, 2, 5, 10, and 20 minutes. TF-1 cell lysates from 2 × 10^6 cells or a controlled amount were equally loaded and separated on a 10% SDS-polyacrylamide gel. Blots were blocked overnight at 4°C after transfer to nitrocellulose (Amer sham; Piscataway, NJ), then probed with antibody against phosphorylated (Ser133) and unphosphorylated CREB (UBI; 1:1,000 and 1:2,000, respectively). Enhanced chemiluminescence (ECL; Amer sham) was used for detection. For identifying phosphorylated (Ser133) and unphosphorylated (Ser133) ERK1 and ERK2 (Santa Cruz; Santa Cruz, CA) for 2 hours at room temperature. Western-Star chemiluminescence (TROPIX; Bedford, MA) was used for detection. Western blots were reprobed with a 1:100 dilution of myosin antibody (Santa Cruz) for controlled loading. Quantification of Western blots was performed by densitometry.

Immunoprecipitation and immune complex kinase assay

TF-1 cells (2 × 10^7 per sample) were serum- and factor-starved, and placed in RPMI + 0.5% BSA for 24 hours before stimulation. Cells were treated with rhGM-CSF (1 nmol/L) for 2, 5, 10, 15, 30, and 60 minutes. Diluent treatment for 10 minutes served as the negative control, and TPA stimulation for 10 minutes as the positive control. Cells were lysed in immune complex kinase (ICK) lysis buffer (10 mmol/L Tris, pH 7.6, 50 mmol/L NaCl, 1% Triton X-100, 30 mmol/L sodium pyrophosphate, 0.1 mmol/L sodium molybdate, 50 mmol/L NaF, 1 mmol/L EGTA, 1 mmol/L sodium vanadate, 5 mmol/L benzamidine, 1% aprotinin, and 1 mmol/L PMSF) and sonicated for 5 seconds. Total soluble protein (600 µg) was incubated with 2 µg of anti-RSK1 antibody (Santa Cruz; specific for pp90RSK1) for 1 to 2 hours at 4°C and precipitated by overnight incubation with protein A/G agarose beads (Santa Cruz). Affinity-purified GST-CREB fusion protein (11 µg; gift from G. Perini) was used as substrate. The fusion protein was isolated by using previously determined conditions. The GST moiety was removed by cleavage, demonstrating phosphorylation of CREB and not GST, thereby indicating that the CREB moiety is phosphorylated. The reaction mixtures totaled 30 µL, which contained 600 µg of immunoprecipitated cell extract (as described above), 30 mmol/L HEPES, pH 8.0, 10 mmol/L MgCl2, 1 mmol/L DTT, 20 µmol/L ATP, 5 mmol/L benzamidine, and .148 MBq P gamma-ATP. Reaction mixtures were incubated for 30 minutes at 30°C. SDS-sample buffer was added to stop reactions. Samples were loaded and electrophoresed on a 10% SDS-polyacrylamide gel, followed by Coomassie blue dye staining. The gel was destained, dried, and autoradiographed. The bands that were detected on the autoradiogram represented phosphorylated GST-CREB. These bands were excised and quantitated by scintillation counting.

Transient transfections

Ten million TF-1 cells (unless otherwise noted) were serum- and factor-starved for 24 hours, as previously described. Transfections were performed by electroporating at 200V with a capacitance of 960 microfarads (µF) (Gene Pulser; Bio-Rad; Hercules, CA). A reporter construct consisting of 116 nucleotides of the egr-1 promoter was subcloned into the pCAT (chloramphenicol acetyl transferase; Promega) vector. The –116 CAT/egr-1 reporter construct containing the CRE represents the minimally active egr-1 promoter. Cells were transfected with the following constructs: (1) RSK, 20 µg; (2) –116 CAT/egr-1, 20 µg; (3) empty control vectors pcDNA3 (Invitrogen; Carlsbad, CA) or pCEP4 (Invitrogen), 10 to 20 µg; (4) CMV β-gal, 4 to 5 µg. T. Fisher and S. Richards (Harvard Medical School) provided the pp90RSK expression constructs. A CMV β-galactosidase assay was used as an internal control for transfection efficiency (Promega). After transfection, TF-1 cells were resuspended in RPMI + 0.5% FCS, then stimulated with diluent control (PBS + 0.02% BSA), rhGM-CSF (1 nmol/L) or TPA (50 ng). TF-1 cells were harvested after 4 hours of stimulation. For MEK inhibitor studies, cells were pretreated with inhibitor PD98059 (20 µmol/L; Calbiochem; San Diego, CA) for 1 hour before stimulation. The lysate was divided equally for CAT and β-galactosidase (β-gal) assays. Hood induction was determined by dividing the percentage of acetylation of stimulated by the percentage of unstimulated samples. Corrected fold induction was calculated by dividing fold stimulation by the ratio of CMV β-galactosidase activity for stimulated versus unstimulated samples. The Student t test was used for statistical analysis (JMP In program).

Results

Expression of pp90RSK in TF-1 cells

pp90RSK has been shown to localize to the nucleus and cytoplasm. Nuclear localization of pp90RSK may be critical for initiating proliferation and signaling in myeloid cells is in excess and dependent on the number of receptor sites available for accepting ligand. The bleomycin-resistant F209 cell line, were cultured in RPMI medium containing 10% fetal calf serum (FCS; HYCLONE; Logan, UT), L-glutamine (2 mmol/L; Sigma; St Louis, MO), gentamicin (0.01 mg/mL; Sigma), and penicillin (100 U/mL); streptomycin (100 mg/mL) at a ratio of 1 U/mL to 1 mg/mL (Sigma). Cells were cultured in nonadherent tissue culture plates (100 × 15 mm) at 37°C and 1% CO2. TF-1 cells were stimulated with rhGM-CSF (1 nmol/L) or TPA (50 ng). TF-1 cells were harvested after 4 hours of stimulation. For MEK inhibitor studies, cells were pretreated with inhibitor PD98059 (20 µmol/L; Calbiochem; San Diego, CA) for 1 hour before stimulation. The lysate was divided equally for CAT and β-galactosidase (β-gal) assays. Hood induction was determined by dividing the percentage of acetylation of stimulated by the percentage of unstimulated samples. Corrected fold induction was calculated by dividing fold stimulation by the ratio of CMV β-galactosidase activity for stimulated versus unstimulated samples. The Student t test was used for statistical analysis (JMP In program).
subcellular localization of pp90RSK in TF-1 cells, extracts were prepared by modified Dignam method.8 Western blot analysis with nuclear or cytoplasmic extracts containing 20 µg of total protein from unstimulated and GM-CSF–stimulated TF-1 cells exhibited a 90-kd protein on probing with anti-RSK1 antibody (UBI) (Figure 1). pp90RSK was consistently observed in both the nucleus and cytoplasm, with the majority of protein present in the nucleus. There seems to be a slight mobility shift on GM-CSF stimulation, which would be consistent with RSK activation. To ensure that these extracts were purely cytoplasmic or nuclear, we performed Western blot analysis probing with antibodies raised against JAK2 and CREB, which are specific for detecting cytoplasmic and nuclear proteins, respectively (data not shown).

pp90RSK phosphorylates cAMP response element-binding protein in granulocyte-macrophage colony-stimulating factor–treated cells

We first determined whether pp90RSK is activated by GM-CSF in TF-1 cells by measuring the ability of RSK to phosphorylate CREB in vitro kinase assays. Immunoprecipitation was performed with anti-RSK1 antibody and TF-1 whole-cell extracts. An affinity-purified GST-CREB fusion protein (63 kd) was used as the substrate. Analysis of the autoradiogram revealed pp90RSK phosphorylation of CREB within 5 minutes of GM-CSF stimulation and maximal phosphorylation at 15 minutes (Figure 2A). Quantitation of phosphorylation by scintillation counting demonstrated a 2.5-fold increase in RSK activity with GM-CSF stimulation (Figure 2B). Levels decreased by 30 minutes after stimulation, and were almost undetectable by 1 hour. Lysates from TF-1 cells treated with diluent (PBS + 0.02% BSA) demonstrated low levels of GST-CREB phosphorylation. Phosphorylation of endogenous CREB in GM-CSF–stimulated TF-1 extracts was also detected on longer exposure (data not shown). TPA has previously been shown to activate CREB and RSK through a protein kinase C (PKC)-dependent pathway,15 and was used as the positive control. Phosphorylation was specific to CREB protein, because an affinity-purified GST-Jun did not demonstrate phosphorylation (data not shown). Jun has been shown to be phosphorylated by a Jun kinase (JNK) but not the ERK signaling cascade.31 These results indicate that GM-CSF–induced pp90RSK activation of CREB occurs within minutes of stimulation of TF-1 cells.

pp90RSK activation is required for granulocyte-macrophage colony-stimulating factor–induced egr-1 expression

Although we demonstrated that pp90RSK is activated by GM-CSF and able to phosphorylate CREB in vitro, these results alone do not provide evidence that phosphorylation may occur in vivo. In an effort to link GM-CSF–induced pp90RSK activation and CREB phosphorylation to egr-1 expression, we performed transfection experiments using the egr-1 reporter construct and kinase-defective pp90RSK (RSK KD). We previously showed that the −116 CAT/egr-1 promoter construct is induced 3-fold in response to GM-CSF stimulation in TF-1 cells, and that the CRE contained within this region is required for maximal transcriptional activation.9 The −116 nucleotide egr-1 promoter construct containing the chloramphenicol acetyltransferase (CAT) gene was cotransfected with expression vectors containing wild-type pp90RSK (RSK WT), kinase-defective (K112/464R) pp90RSK, or empty vector control pcDNA3. No significant differences in GM-CSF–induced reporter activity were observed on cotransfection of the −116 CAT egr-1 promoter construct with the wild-type pp90RSK (2.81 ± 0.74) in comparison to the pcDNA3 vector alone (3.17 ± 0.43) (Figure 3). The expression of CAT activity from the −116/egr-1 promoter construct does not increase with overexpression of RSK WT as

Figure 1. Expression of pp90RSK in TF-1 cells. Unstimulated or GM-CSF–stimulated TF-1 cell nuclear (N) and cytoplasmic (C) extracts were isolated by a modified Dignam procedure.9 Protein (20 µg) was separated on a 10% polyacrylamide gel. The blot was probed with 3 µg/mL of pp90RSK antibody (UBI). ECL was used for detection. The arrow indicates pp90RSK protein. These results were confirmed in 2 independent experiments.

Figure 2. GM-CSF stimulates pp90RSK activation and CREB phosphorylation. TF-1 cells (2 × 10⁶ per time point) were serum- and factor-starved for 24 hours, and stimulated with mGM-CSF (1 nmol/L) for 2, 5, 10, 15, 30, and 60 minutes, and with diluent for 10 minutes as negative control (BSA + 0.02% in PBS). TPA (50 ng/mL) served as the positive control. Cell lysate (600 µg) was immunoprecipitated with anti-RSK antibody (2 µg per sample). (A) Immune complex kinase assays were performed with affinity-purified GST-CREB (11 µg per sample). The arrow indicates the location of GST-CREB (63 kd). (B) Phosphorylation was quantitated by scintillation counting. The results represent 1 of 3 separate experiments.

Figure 3. pp90RSK activation is required for maximal transcription of the −116 CAT/egr-1 promoter construct. TF-1 cells (10⁷) were factor- and serum-starved for 24 hours, and placed in serum-free media. Twenty micrograms of −116 CAT/egr-1 construct, 20 µg of wild-type pp90RSK, and 20 µg of kinase-defective pp90RSK were electroporated into TF-1 cells and stimulated with either diluent control (PBS + 0.02% BSA) or mGM-CSF (1 nmol/L) for 4 hours. Four micrograms of CMV-β-galactosidase plasmid was cotransfected as the internal control for transfection efficiency. Fold induction represents percentage acetylation (by CAT assay) of constructs stimulated by GM-CSF divided by percentage of diluent-stimulated constructs. P values were determined by Student paired t test analysis. Data represent the average of 3 independent experiments; each transfection was performed in triplicate.
demonstrated in Figure 3. The fold stimulation with RSK WT is slightly less than vector. However, a statistically significant difference in fold induction was observed between the vector control and the kinase-defective pp90RSK (1.81 ± 0.33; P = .043). The percentage acetylation of diluent-treated cells in cotransfections with −116 CAT and RSK KD or vector was not statistically different (P = .2 and .083, respectively; data not shown). We determined the level of expression of RSK WT and RSK KD in the cotransfected cells by performing Western blot analysis. There is approximately a 3-fold increase in RSK protein expression when comparing either the RSK WT or RSK KD transfected cells to that of the empty control vector (data not shown). We would not expect to see a dramatic difference in protein expression levels because of low transfection efficiency (10%-30%) after transient transfection. Therefore, pp90RSK is a critical kinase responsible for phosphorylating CREB and activating egr-1 expression in the GM-CSF signal transduction pathway.

Granulocyte-macrophage colony-stimulating factor induces phosphorylation of ERK 1 and ERK2

We previously demonstrated that GM-CSF activates MAPKs in HL60 cells and neutrophils.32 Because pp90RSK phosphorylation can be mediated by a MEK/ERK pathway, we examined phosphorylation of MAPKs ERK1 and ERK2 in TF-1 cells stimulated by GM-CSF. In these experiments, phospho-specific antibodies were used to detect ERK1 and 2 activation.32 Treatment of TF-1 cells with GM-CSF caused a rapid and potent increase in ERK activity. A significant increase was observed within 2 minutes, and peaked at 10 minutes after stimulation (Figure 4A). ERK activation by GM-CSF then diminished by 20 minutes after stimulation. Maximal activity occurred at a biologically relevant concentration of GM-CSF. Western blots with unphosphorylated ERK antibody revealed constant levels of ERK1 and ERK2 protein throughout the time course, indicating that ERK levels are unchanged (Figure 4B).

An MEK-dependent signaling pathway is necessary for transcriptional activation of egr-1 and phosphorylation of ERKs, pp90RSK and cyclic adenosine monophosphate response element-binding protein

MEK has previously been shown to activate ERK1 and ERK2.33-35 To determine whether the GM-CSF signal is transduced through a MEK-dependent pathway, transient transfection assays with the −116 CAT/egr-1 reporter construct in GM-CSF–stimulated TF-1 cells were performed in the absence or presence of a kinase specific inhibitor, PD98059. Alessi et al36 demonstrated that PD98059 specifically inhibits MEK. We performed transient transfections using varying amounts of inhibitor to determine optimal conditions. A concentration of 20 µmol/L PD98059 was found sufficient for inhibition, but not toxic to the cells. Our data demonstrated that there was a statistically significant decrease in fold induction in cells treated with 20 µmol/L of PD98059 (1.0-fold; P = .0114) in comparison to untreated cells (2.0-fold) (Figure 5A). TPA was used as the positive control, because we and others have previously shown that egr-1 is also induced by a protein kinase C (PKC)-dependent pathway.9,12 As expected, there was no statistically significant difference in fold induction on TPA stimulation in the presence of the MEK inhibitor (Figure 5B; P = .6185). Percentage acetylation of basal levels in cells treated with diluent was equal in the presence or absence of PD98059 (data not shown). This is consistent with our hypothesis that GM-CSF stimulates egr-1 expression through a MEK-dependent pathway.

To demonstrate that PD98059 inhibits phosphorylation of ERK, we performed Western blot analysis with extracts from treated and untreated TF-1 cells and antiserum raised against phosphorylated ERK1 and 2. Reduced levels of phosphorylated ERKs were observed in the presence of PD98059 in GM-CSF–stimulated cell extracts (Figure 6A), as well as on probing with antiphosphorylated CREB antibody (Figure 6B). Thus, a significant decrease in phosphorylation of ERK and CREB in response to GM-CSF in the presence of the MEK inhibitor (20 µmol/L) is observed. Weak CREB phosphorylation in response to GM-CSF in the presence of GM-CSF is demonstrated; however, the important feature of Figure 6B is the comparison between the blots with and without PD98059 inhibitor. In contrast, TPA-induced activation of ERK1, ERK2, and CREB was not completely inhibited by PD98059, because signaling occurs primarily through PKC. Equal amount of protein was loaded onto the gel as determined by reprobing blots with myosin (data not shown). Our results suggest that RSK1 activation and phosphorylation of ERK1/2 and CREB activation are inhibited in the presence of PD98059.
CREB induces transcriptional activation of egr-1, at least in part, through a MEK-dependent pathway.

Discussion

Several lines of evidence suggest that RSK1 is the kinase responsible for growth factor-induced phosphorylation of CREB. NGF, EGF, and c-Kit have been shown to signal through Ras to activate the ERK pathway and pp90RSK. In addition, ionizing radiation induces activation of pp90RSK in human U-937 myeloid leukemia cells. We demonstrated that GM-CSF is also capable of activating pp90RSK in TF-1 cells. GM-CSF regulates the proliferation and maturation of myeloid progenitor cells and enhances the function of differentiated myeloid cells. Activation of several signaling molecules, including JAK2, Stat1, Stat3, Stat5, Ras, Raf, and ERK, results in induction of growth-related genes; eg, c-fos, c-myc, or egr-1. Others have demonstrated that activation of pp90RSK results in phosphorylation of S6 and serum response factor (SRF) peptides, leading to induction of egr-1 in pokeweed mitogen-treated B cells. Activation of egr-1 transcription and pp90RSK has been observed during induction of monocytic differentiation. We and others have previously demonstrated that maximal transcriptional activation of egr-1 in response to GM-CSF requires CREB phosphorylation at serine 133. The kinase responsible for CREB phosphorylation in response to GM-CSF has not been identified. We propose that GM-CSF induces the activation of RSK1 in part through a MEK-dependent pathway, resulting in phosphorylation of CREB and egr-1 transcription in TF-1 cells.

We show that pp90RSK is present in the human factor-dependent cell line, TF-1. pp90RSK is observed in both the nuclear and cytoplasmic compartments, although most of the protein is found in the nucleus, regardless of stimulation by GM-CSF. Therefore, we have established the presence of both pp90RSK and CREB in the nuclei of TF-1 cells. This is consistent with previous reports demonstrating that pp90RSK appears to undergo nuclear translocation, followed by phosphorylation of potential nuclear targets. Furthermore, no differences in pp90RSK protein expression were observed after GM-CSF treatment of TF-1 cells, which is similar to our previous finding that CREB levels are constant after GM-CSF stimulation. Because egr-1 transcription does not require new protein synthesis, we would not expect pp90RSK expression to increase. Thus, the observed increase in CREB phosphorylation must result from increased pp90RSK phosphorylation and activity rather than an increase in pp90RSK protein synthesis.

In accordance with other reports of CREB phosphorylation by pp90RSK, we hypothesized that pp90RSK phosphorylates CREB in response to GM-CSF in myeloid cells. Our in vitro kinase assays demonstrated that the endogenous pp90RSK in TF-1 cells is capable of phosphorylating recombinant (Figure 2A) and endogenous CREB (data not shown). We have previously shown that CREB is phosphorylated within 5 minutes of TF-1 stimulation. Other cytokines, including heparocyte growth factor, stem cell factor and fibroblast growth factor, induce phosphorylation of serine 133 within the kinase-inducible domain of CREB. This phosphorylation has been proposed to be due to activation of pp90RSK and pp70S6k.

We found that GM-CSF treatment resulted in a 3.0-fold increase in CREB phosphorylation by pp90RSK compared with dimethylsulfoxide-treated cells. This is comparable to what others have reported with RSK in GM-CSF-stimulated neutrophils (1.5-fold). However, only when an in-gel kinase assay was performed against the RSK peptide substrate did a more significant increase occur (3.4-fold). Therefore, this difference in fold induction may be due to the nature of the substrate used for measuring kinase activity (either pp90RSK peptide substrate or GST-CREB) or differences in assay conditions. Our cotransfection experiments with the −116 CAT/egr-1 construct and RSK expression vectors (Figure 3) provide additional evidence that pp90RSK is a functionally relevant kinase activated by the GM-CSF signal transduction pathway in TF-1 cells. On cotransfecting with RSK WT, we did not observe any increase in −116 CAT/egr-1 transcriptional activity possibly because of rate-limiting substrates or kinases. This is similar to our earlier studies that demonstrated that overexpression of WT CREB does not increase transcription of −116 CAT/egr-1 in response to GM-CSF stimulation, possibly for the same reason. The kinase-defective pp90RSK inhibited but did not completely abolish GM-CSF–induced transcriptional activation of −116 CAT, suggesting that other signaling pathways may be involved in the transcriptional activation of egr-1.

Induction of egr-1 transcription by cytokines and serum is mediated through activation of several transcription factors such as CREB and SRF by the Ras/MEK pathway. ERK is phosphorylated, resulting in activation of pp90RSK. Inhibitor studies with PD98059 demonstrated that activation of ERK and CREB in response to GM-CSF is MEK-dependent. Furthermore, transcriptional activation of the −116 CAT/egr-1 promoter construct also appears to be regulated by a MEK-dependent pathway. The varying findings from Figures 5 and 6 are a result of measuring 2 different endpoints: the −116 promoter-driven CAT expression and the phosphorylation of CREB, respectively. According to Pende et al., the MEK inhibitor not only prevents activation of the MAPK signaling pathway, but also strongly decreases CREB phosphorylation by TPA in addition to other stimuli in cortical oligodendrocyte progenitor cells. Therefore, PD98059 may inhibit TPA activation of MEK if there is cross talk between MEK-dependent and PKC-dependent pathways. Consequently, the difference in the effects of PD98059 between Figures 5 and 6 in myeloid cells is not surprising. Furthermore, MEK-independent pathways may activate −116 CAT/egr-1 transcription through other DNA binding sites, such as SRE. Our results are consistent with other studies demonstrating that pp90RSK phosphorylates CREB downstream of Ras in PC12 cells. However, the MEK/MAPK pathway partially mediates CREB regulation by PKC and growth factor activation. Thus, it appears that the MEK-RSK-CREB pathways are conserved in signaling pathways activated by both nontyrosine kinase receptors and receptor tyrosine kinases. Previous reports have characterized the role of pp90RSK during signal transduction downstream of receptor tyrosine kinases such as NGF, EGF, and c-Kit. We demonstrate that pp90RSK links signals from the nontyrosine kinase containing GM-CSF receptor to transcriptional events in the nucleus.

Scheid and Duronio have demonstrated that phosphatidylinositol 3-kinase (PI3K) activity plays an important role in cell survival in hematopoietic cells. Recently, phosphorylation of BAD (Bcl-2/Bcl-X−−associated death promoter), on stimulation by interleukin 3 (IL-3) and platelet-derived growth factor (PDGF), has been demonstrated to be potentially PI3K/PKB (protein kinase B)–dependent. BAD is an apoptotic protein that promotes cell survival or death, depending on its phosphorylation state. Furthermore, the MEK signaling pathway was shown to contribute to the phosphorylation of BAD in the absence of PI3K activity. Another
study by Scheid et al.\(^7\) demonstrated that IL-3–stimulated CREB phosphorylation occurs through 3 possible signaling pathways in myeloid cells: (1) p38MAPK and MAPKAP-Kinase2, (2) cAMP-dependent protein kinase, and (3) a yet unidentified pathway. Clearly, not all of the signaling pathways that involve CREB phosphorylation have been elucidated.

Recent studies have shown that CREB may also play a role in cell survival.\(^8,9\) On the basis of our data, we hypothesize that phosphorylation of pp90RSK and CREB may inhibit apoptosis and promote survival in response to GM-CSF; however, further studies are required to verify this.

Our results suggest that CREB is the substrate for pp90RSK in response to GM-CSF stimulation. With the use of an inhibitor specific for MEK, we were able to demonstrate a MEK-dependent GM-CSF signaling pathway, which ultimately leads to transcriptional activation of the immediate early gene, egr-1. Therefore, signaling pathways induced by both tyrosine kinase and, in this particular case, nontyrosine kinase receptors, may cause activation of pp90RSK and subsequent phosphorylation of critical transcription factors in response to growth factor stimulation.

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References


46. del Peso L, Gonzalez-Garcia M, Page C, Herrara R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science. 1997;278:687-689.


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