Granulocyte-Macrophage Colony-stimulating Factor Induces the Transcriptional Activation of egr-1 through a Protein Kinase A-independent Signaling Pathway*

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) rapidly and transiently induces the transcriptional activation of the early growth response gene-1 (egr-1) in the human factor-dependent myeloid leukemic cell line, TF-1. We previously demonstrated that the cAMP response element (CRE) is required for GM-CSF-induced egr-1 expression and that phosphorylation of CREB on serine 133 plays a critical role during GM-CSF signal transduction. To determine whether GM-CSF activates signaling pathways through a protein kinase A-dependent or -independent pathway, we measured cAMP levels following GM-CSF or forskolin treatment of TF-1 cells. Forskolin but not GM-CSF stimulation resulted in an increase in cAMP levels. Transient transfection assays with TF-1 cells were also performed with a 116-nt nucleotide egr-1 promoter construct and the protein kinase inhibitor, PKI. Although PKI inhibited forskolin induction of the 116-nt nucleotide construct, it did not affect GM-CSF stimulation of this construct. In the present study, we demonstrated that GM-CSF induces egr-1 expression through a protein kinase A-independent pathway.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates the proliferation and maturation of myeloid progenitors and enhances the function of differentiated effector cells (1–3). The biological activities of GM-CSF are mediated by a heterodimeric receptor which consists of an α and β subunit. The β subunit is critical for signal transduction, but does not contain intrinsic tyrosine kinase activity (4, 5). The interaction of GM-CSF and its receptor results in the activation of a number of signaling molecules, including JAK2, Ras, Raf, and mitogen-activated protein kinase (4, 6, 7). Phosphorylation of several of these kinases leads to induction of the growth-related genes, c-fos, c-myc, and egr-1 (4, 8). The link between cytoplasmic events and the activation of specific transcription factors in the nucleus has not been studied extensively.

We previously demonstrated that the induction of the immediate early gene, egr-1, is rapid, transient, and independent of protein synthesis (9). Transient transfections of egr-1 promoter constructs in the human factor-dependent myeloid leukemic cell line, TF-1, showed that the cAMP response element (CRE) located between nucleotides −57 and −76 was required for transcriptional activation in response to GM-CSF (9). We also demonstrated that the CRE-binding protein, CREB, associates with the CRE in the −116-nt region of the promoter and is phosphorylated on serine 133 in GM-CSF-stimulated cells (10). This phosphorylation of CREB is critical for GM-CSF-induced egr-1 expression.

The mechanism of CREB phosphorylation on serine 133 has been shown previously to be mediated through a protein kinase A-dependent pathway (11–13). Recently, CREB has been demonstrated to be activated by a PKA-independent pathway (14). To determine whether GM-CSF signaling results in activation of CREB through a PKA-dependent or -independent pathway in TF-1 cells, we measured cAMP levels in cells stimulated with GM-CSF. We further examined the effects of the protein kinase A inhibitor, PKI, on the transcriptional activation of egr-1. PKI is a potent inhibitor of the catalytic subunit of the PKA-dependent protein kinase. Overexpression of the cloned human PKI has been shown to inhibit protein kinase A activity (15–17). Transient co-transfection assays were performed in TF-1 cells with an expression plasmid containing PKI and a 116-nt construct, which contains the CRE site. Our results demonstrate that GM-CSF stimulation does not increase cAMP levels and that the transcriptional activation of egr-1 occurs through a PKA-independent pathway.

MATERIALS AND METHODS

Cell Culture—TF-1 cells were cultured at 37 °C in RPMI with 10% fetal calf serum, 1-glutamine (2 mM), penicillin (100 units/ml)/streptomycin (100 mg/ml) at a ratio of 1 unit/ml to 1 mg/ml, and rbGM-CSF (500 pu) in nonadherent tissue culture plates.

cAMP Assays—TF-1 cells were factor- and serum-starved and placed in RPMI with 0.5% bovine serum albumin (BSA) for 24 h. Cells (107/sample) were transferred to a Microfuge tube and stimulated with 1, 2, 5, 10, and 15 min for GM-CSF; and 5, 10, and 15 min for forskolin (5). They were then incubated in a 37 °C shaking water bath for varying lengths of time (10 min for diluent control; 1, 2, 5, 10, and 15 min for GM-CSF; and 5, 10, and 15 min for forskolin). After incubation, cells were spun at 14,000 rpm for 5 s at room temperature. Cells were lysed with 60% ethanol, incubated for 30 min at room temperature, and centrifuged at 14,000 rpm for 10 min at room temperature. The supernatant was collected and dried in a vacuum centrifuge (Speed Vac) for 1 h. The pellet was resuspended in 150 μl of Tris-CI, 0.05 M, pH 7.5, EDTA, 4 mM. The amount of cAMP in each sample was then determined using the cAMP assay system (Amerham), which is based on the competition between unlabeled cAMP in the cell extract and a fixed quantity of [3H]-labeled cAMP to bind to a protein that has a high specificity and affinity for cAMP. The amount of [3H]cAMP-protein complex formed is inversely related to the amount of unlabeled cAMP. Measurement of the protein-bound radioactivity enables the amount of cAMP in our sample to be calculated.

Transient Transfection Assays—TF-1 cells were factor- and serum-starved for 24 h and placed in RPMI, 0.5% BSA (Sigma). A total of 105
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**RESULTS AND DISCUSSION**

**GM-CSF Stimulation Does Not Increase cAMP Levels in TF-1 Cells**—To determine whether GM-CSF stimulation of TF-1 cells resulted in an increase in cAMP levels, factor- and serum-starved cells were stimulated with GM-CSF (1 nm) for 1, 5, 10, and 15 min and then harvested. Forskolin (10 μM), a known activator of protein kinase A, was used as the positive control. TF-1 cells were stimulated with the diluent control (PBS, 0.02% BSA) for 10 min, representing the zero time point. Compared to forskolin, GM-CSF stimulation did not result in an increase in cAMP levels (Fig. 1). Forskolin stimulation resulted in a 2.3- to 2.4-fold increase in cAMP levels at the 5-, 10-, and 15-min time points (p < 0.05, Fig. 1 and Table I). GM-CSF stimulation produced a 1.2-fold increase in cAMP levels at 1 min; however, this was not statistically significant (p = 0.06). The y axis represents the ratio of picomoles of cAMP/tube in forskolin- or GM-CSF-stimulated cells to the picomoles of cAMP/tube in diluent-treated cells. The data for each time point shown in Table I represent the average of three to five independent experiments performed in duplicate. Time points beyond 15 min might be a reflection of secondary events that depend on protein synthesis instead of immediate events in response to GM-CSF signaling. The fold induction we observed is consistent with the findings reported previously (15). Our data therefore demonstrate that although TF-1 cells are capable of responding to activators of protein kinase A (i.e. forskolin), the interaction of GM-CSF with its cognate receptor does not activate protein kinase A by a cAMP-dependent pathway. The cAMP levels were measured to 15 min, since induction of egr-1 occurs within 15–30 min following GM-CSF stimulation (8, 9).

**The Effect of the Protein Kinase A Inhibitor, PKI, on the Transcriptional Activation of egr-1 in GM-CSF-stimulated TF-1 Cells**—We previously utilized the –116-nt egr-1 promoter construct to demonstrate that CREB activates transcription through its interaction with nucleotides, including –57 to –76, which contain the CRE. This element is necessary but not sufficient for GM-CSF-induced egr-1 expression (9). Transient co-transfections performed with the –116-nt construct and an expression vector containing CREB, or CREB mutated on serine 133 to alanine, demonstrated that the phosphorylation of CREB is required for transcriptional activation of egr-1 (10). To determine the effects of protein kinase A inhibition on transcriptional activation of egr-1 in response to GM-CSF, we transiently co-transfected a construct containing the PKI in an expression vector and the –116-nt construct. The CMV β-galactosidase plasmid was used as the internal control for transfection efficiency.

| Stimulus | Incubation | cAMP | Stimulation | p value |
|----------|------------|------|-------------|---------|
| Diluent  | 10         | 1.96 ± 0.147 | -fold      |         |
| GM-CSF   | 1          | 2.39 ± 0.097 | 1.2 ± 0.08 | 0.06    |
|          | 2          | 2.06 ± 0.085 | 1.0 ± 0.08 | 0.682   |
|          | 5          | 1.60 ± 0.289 | 0.85 ± 0.03| 0.314   |
|          | 10         | 1.58 ± 0.130 | 0.87 ± 0.05| 0.682   |
| Forskolin| 5          | 4.64 ± 0.900 | 2.3 ± 0.27 | < 0.05  |
|          | 10         | 4.70 ± 0.427 | 2.4 ± 0.16 | < 0.05  |
|          | 15         | 4.42 ± 0.245 | 2.3 ± 0.42 | < 0.05  |

Our experiments demonstrated that the –116-nt construct transfected with the vector control, pcDNA3, resulted in a 4.0-fold induction in response to GM-CSF stimulation (Fig. 2). When the –116-nt construct was co-transfected with the PKI construct, there was no difference in fold induction in response to GM-CSF in three independent experiments (p = 0.748). The difference in the fold induction between pCAT or the –116-nt construct co-transfected with the vector pcDNA3 was significant (p < 0.05). In response to forskolin stimulation, a 2.1-fold induction of the –116-nt construct was observed (Fig. 2). Although this fold induction was approximately half of that seen with GM-CSF, the value was found to be statistically significant (p < 0.05) compared to that of the vector pCAT. When the –116-nt construct was co-transfected with the PKI plasmid and stimulated with forskolin, a statistically significant decrease in fold induction was also observed compared to the pcDNA3 vector (p < 0.05, Fig. 2). These data represent an average of two to four separate experiments, with each transfection performed in duplicate. The low degree of stimulation by forskolin may be due to the fact that a single CRE in the –116-nt construct was not sufficient to activate transcription more than 2-fold. Our previous results demonstrated that the serum response element contained within the –116-nt region is required for GM-CSF-induced transcriptional activation of egr-1. Cooperation between CREB and serum response element-binding proteins may enhance interaction with the tran-

\[ ^2 \text{M. Uhler, personal communication.} \]
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Fig. 2. PKI inhibits the transcriptional activation of egr-1 by forskolin but not by GM-CSF. TF-1 cells (106) were factor- and serum-starved for 24 h and placed in serum-free media. Twenty micrograms of reporter construct −116cat and 22 μg of PKI were electroporated into TF-1 cells and stimulated with diluent control (0.02% BSA in PBS), GM-CSF (1 nm), or forskolin (10 μM) for 4 h. Three micrograms of CMV β-galactosidase plasmid was co-transfected as the internal control for transfection efficiency. CAT or β-galactosidase assays were performed. Fold induction represents the percent acetylation of constructs stimulated with GM-CSF or forskolin divided by the percent acetylation of constructs stimulated with diluent control. p values were determined by Student’s t test analysis. These data represent three independent experiments, with each transfection performed in duplicate.

scriptional machinery, explaining the increase in stimulation by GM-CSF.

We also examined the effect of PKI on the basal activity of the −116cat construct. The basal activity represents the percent acetylation of cells treated with the diluent control (PBS, 0.02% BSA). The presence of PKI affected the basal activity of −116cat (data not shown). PKI, however, did not affect the general transcriptional activity in TF-1 cells or produce non-specific inhibition of cellular processes, since the CMV β-galactosidase activity was not significantly different in cells transfected with vector compared to PKI. Our results demonstrated that GM-CSF does not increase cAMP levels in TF-1 cells and that the presence of PKI does not affect the transcriptional activity of −116cat in response to GM-CSF. Although PKI affected the basal activity of −116cat, the fold induction and the β-galactosidase activity were not affected and were consistent with our previously published data (9, 10).

The present findings suggest that egr-1 transcriptional activation by CREB in response to GM-CSF is mediated by a protein kinase A-independent pathway. Previously published reports studying the role of adenylate cyclase and cAMP during GM-CSF signal transduction have been inconsistent and cell type-dependent. Studies with macrophages and bone marrow progenitor cells have shown that GM-CSF can elevate intracellular levels of cAMP and activate PKA (18, 19). Furthermore, GM-CSF enhances the metastatic phenotype of lung carcinoma cells through a protein kinase A-dependent pathway (20). However, in the mouse mast cell line, PT18, GM-CSF did not alter the activity of cAMP-dependent protein kinase or protein kinase C (21).

Interestingly, our results are consistent with studies that show a decrease in cellular cAMP levels, which is associated with stimulation of HL-60 proliferation, while elevations in cyclic nucleotides are related to an inhibition of HL-60 proliferation and potentiation of differentiation (22). Since GM-CSF stimulates TF-1 cell proliferation, our findings are in accordance with this hypothesis.

Recently, a novel CREB kinase has been identified as phosphorylating CREB on serine 133 through a protein kinase A-independent pathway (14). Nerve growth factor stimulates the differentiation of PC12 cells by activating Ras-dependent pathways, resulting in CREB phosphorylation and induction of c-fos. Furthermore, p90RSK was shown to phosphorylate CREB on serine 133 in melanoma cells treated with fibroblast growth factor (23). Our results suggest that a novel CREB kinase or a previously unknown serine/threonine kinase such as p90RSK or p70s6 may be one of the candidate kinases responsible for activation of CREB during GM-CSF signal transduction. Further studies to identify the kinase-activating CREB will provide a link between the signals from the cytoplasm to the nucleus that induce growth-related genes.

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