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**βc Cytokine Receptor-Induced Stimulation of cAMP Response Element Binding Protein Phosphorylation Requires Protein Kinase C In Myeloid Cells: A Novel Cytokine Signal Transduction Cascade**

Elaina Gubina, Xu Luo, E. Kwon, Kathleen Sakamoto, Yu Fang Shi, and R. Allan Mufson

We have recently shown that IL-3R occupancy activates a phosphatidylcholine-specific phospholipase C, and the sustained diacylglycerol accumulation subsequently activates protein kinase C (PKC). In human IL-3-dependent myeloid cells (TF-1), the novel PKCe isoform regulates bcl-2 expression and cell survival. The report of a PKC activatable cAMP response element (CRE) in the bcl-2 promoter and a role for PKC in bcl-2 expression in B cells led us to the hypothesis that PKC phosphorylation activates transcription factor CREB after IL-3R engagement. We found that IL-3 and GM-CSF induced phosphorylation of CREB on Ser133 in TF-1 cells, and this phosphorylation was blocked by two structurally unrelated classes of PKC inhibitors. An inhibitor of cyclic nucleotide-dependent kinases did not block this phosphorylation. IL-4, which is biologically active in these cells but does not use the βc common subunit, did not phosphorylate CREB on Ser133. Inhibition of mitogen-activated protein kinase kinase (MEK) blocks CREB phosphorylation. The PKC inhibitors, but not a cyclic nucleotide-dependent kinase inhibitor, blocked IL-3 activation of CRE-dependent transcription from an *egr-1* promoter/chloramphenicol acetyltransferase (CAT) reporter construction transiently transfected into TF-1 cells. Finally, TF-1 cells stably overexpressing PKCe, but not the δ isoform of PKC, enhanced CRE-dependent CAT expression from the promoter/reporter construct. Therefore, it is likely that a PKCe kinase cascade resulting in CREB phosphorylation represents a novel signal transduction cascade for regulating cellular gene expression through the βc common cytokine receptor. The *Journal of Immunology*, 2001, 167: 4303–4310.

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3 E.G. and X.L. made equally significant contributions to this paper.

4 Abbreviations used in this paper: PKC, protein kinase C; CRE, cAMP response element; MAP, mitogen-activated protein; CAT, chloramphenicol acetyltransferase; PC-PLC, phosphatidylcholine-specific phospholipaseC; PKA, protein kinase A; CBP, CREB binding protein; CaMK, calmodulin kinase; SRE, serum response element; kₐ, association rate constant; TPA, 12-O-tetradecanoyl phorbol-13-acetate; JAK, Janus kinase; ERK, extracellular signal-regulated kinase; MAPK, MAP kinase; rsk, etc.
been identified in the egr-1 gene promoter, and GM-CSF activation of this promoter required CREB phosphorylation on Ser133 (7). Recently, a positive regulatory region has been found in the bel-2 promoter, and this region contains a cAMP response element (CRE) consensus sequence (GTGACGTCA) (8). Expression of bel-2 in B cells is dependent on this promoter element and phosphorylation of the associated trans-activating factor CREB, although participation of other upstream regulatory elements was also required. GM-CSF-induced CREB phosphorylation is independent of activation of the cAMP/GMP-dependent kinases (7). CREB is resident in the nucleus, but its binding to CREB binding protein (CBP) is dependent on the phosphorylation of Ser133 in the transactivation domain of CREB. The interaction of CREB/CBP with transcriptional complexes presumably initiates transcription from CRE-containing genes (9). Initially this phosphorylation was determined to be PKA-dependent, and later calmodulin kinase (CaMK) was also implicated in CREB phosphorylation (10). In B lymphocytes, cross-linking of surface Ig with anti-Ig Ab triggers (CaMK) was also implicated in CREB phosphorylation (10). In B lymphocytes, cross-linking of surface Ig with anti-Ig Ab triggers CREB Ser133 phosphorylation (11). Interestingly, however, this Ig-induced phosphorylation was determined to be independent of PKA or CaMK, but appeared to depend on PKC-mediated phosphorylation.

It thus seemed possible that CREB could be a downstream transcription factor responsive to βCR-mediated PKC activation, and an important effector in a novel βCR cytokine signal transduction cascade regulating growth and survival genes. We have found in TF-1 cells that active PKC is required for CREB Ser133 phosphorylation in response to βCR cytokines. In addition, we have determined that PKC is required for βCR cytokine stimulation of CREB-dependent transcription in these cells. Finally, using TF-1 cell lines stably overexpressing PKC isofrom ε, we have shown that a specific novel isofrom of PKC is responsible for stimulating CREB-dependent transcription.

Materials and Methods
Reagents and DNA vectors
Purified recombinant human cytokines were purchased from BioSource International (Camarillo, CA). Protein kinase inhibitors H-7 and HA1004 were purchased from Biomol (Plymouth Meeting, PA). The PKC inhibitor bisindolylmaleimide I was purchased from two sources. The bisindolylmaleimide purchased from L.C. Laboratories (Woburn, MA) is designated GF109203X, and from Calbiochem (San Diego, CA) it is designated GO6908. The inhibitor from both sources gave exactly the same results. The PKC-specific hexamidine inhibitor NPC15432 and the mitogen-activated protein (MAP) kinase (MAPK) kinase inhibitor PD98059 were purchased from L. C. Laboratories. Specific Abs recognizing CREB or CREB phosphorylated on Ser133 were purchased from Upstate Biotechnology (Lake Placid, NY). The anti-phosphoCREB Ab was a rabbit anti-peptide Ab that was made against residues 123–126 of rat CREB and is specific only for phosphorylated CREB. This anti-phosphoCREB Ab recognizes both rat and human phosphoCREB. The anti CREB Ab is a rabbit Ab made against amino acids 5–24 of human CREB. The Ab does not recognize phosphoCREB. The egr-1 promoter/chlororaphenicol acetyltransferase (CAT) reporter constructions used in the transient transfection studies have been described previously (7). Briefly, the 116 enhancer construction is composed of a CRE, serum response element (SRE), and TATTA box promoter linked to a CAT reporter gene, 116 M, which was the 116 construction with the CRE deleted, and S6, which had both enhancer elements deleted and contained only the promoter (7). Previous work using EMSAs and transient transfection assays has shown that cytokine activation of this enhancer/promoter construction is dependent on activation of the CRE and the presence of CREB in transcription complexes.

Cell culture
Wild-type TF-1 cells or stably transfected TF-1 cell lines were routinely passaged in RPMI 1640 medium supplemented with 10% FCS and 10% A5637 bladder carcinoma-conditioned medium as a source of cytokines. Derivation of cell lines stably overexpressing PKC isofroms was previously described (6). PKC-transfected cell lines were periodically assayed for appropriate isofrom overexpression by immunoblotting. Protein kinase inhibitors were used at 5– to 10-fold of the in vitro association rate constant (k) values for enzyme inhibition as recommended by the manufacturers and the open literature.

Immunoblotting
Immunoblotting was performed as described previously (4, 6). Briefly, cell lysates were prepared and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose filters and the filters were probed with appropriate Abs. The anti-P Ser133 CREB Ab was used first, and then the blots were stripped and reprobed with the anti-CREB Ab. Probed blots were developed with ECL reagents (Amersham, Little Chalfont, England), and the developed blots were exposed to Kodak (Rochester, NY) X-AR film.

Transient transfection
TF-1 cells were deprived of cytokine, but maintained in serum for 12 h before transfection. Aliquots of 105 cells were electroporated with 10 μg of egr-1 promoter/CAT reporter plasmid DNA and 10 μg of control plasmid containing β-galactosidase driven by a CMV promoter in RPMI 1640 medium without serum or antibiotics as previously described (6). After electroporation, cells were incubated for 10 min on ice. The cells were then transferred into 10 ml of fresh serum-supplemented RPMI 1640 medium in the absence or presence of different combinations of cytokines and inhibitors. The cells were continually maintained in the presence of serum to obviate any serum stimulation in the experiments. Cells were incubated at 37°C for 6 h in a humidified CO2 incubator before lysis for enzyme assay. All transfections were performed in triplicate, and triplicate CAT and β-galactosidase assays were conducted for each sample to correct for variation in transfection efficiency.

CAT activity assay
CAT activity was measured using an enzyme assay system obtained from Promega (Madison, WI). Briefly, cells were washed with PBS and resuspended in 300 μl of lysis buffer. After a 10-min incubation at 60°C, lysates were centrifuged. Aliquots of supernatant (25 μl) were collected and mixed with 5 μl of n-butyrylCoA containing 0.5 μCi of [3H]chlororaphenicol. The assay volume was adjusted to 125 μl with 0.25 M Tris, pH 8.0. The assay mixture was incubated at 37°C for 24 h. The organic fraction was extracted with xylene, and radioactivity was determined by liquid scintillation counting. All CAT activities were corrected for transfection efficiency as determined from results with the control β-galactosidase plasmid.

Results
Inhibitors of the PKC catalytic domain block IL-3 induction of CREB phosphorylation
We began by examining the effect of IL-3 stimulation on CREB phosphorylation in factor-deprived TF-1 cells. Cells were deprived of cytokine for 12 h and restimulated with 10 ng/ml recombinant human IL-3. Lysates from stimulated and unstimulated cells were separated by PAGE and transferred to nitrocellulose. Blots were probed with a rabbit Ab specifically recognizing human CREB phosphorylated on Ser133. A very low level of CREB phosphorylation was observable in the factor-deprived cells, and the addition of IL-3 induced a strong increase in phosphorylation at 15 min (Fig. 1). The induced level of phosphorylation had declined significantly by 30 min. It has already been demonstrated that activation of the βCR does not induce changes in cAMP levels in TF-1 cells, IL-60 cells, or primary eosinophils (7, 12, 13). In addition, PKA inhibitors do not inhibit GM-CSF-induced CREB phosphorylation (12, 14). Thus, a cyclic nucleotide-independent protein kinase must be responsible for CREB phosphorylation in βc cytokine-dependent cells. When TF-1 cells were preincubated for 5 min with 30 μM preferential PKC inhibitor H-7 and stimulated with IL-3 in the presence of this inhibitor, the Ser133 phosphorylation of CREB was completely abrogated. A similar incubation with an equimolar concentration of the structurally related preferential PKA inhibitor HA1004 had no effect on IL-3 stimulation of Ser133 phosphorylation. H-7 has a k for PKC that is almost 1 log lower than HA1004, but they inhibit PKA equivalently. Therefore,
FIGURE 1. Effect of PKC catalytic domain inhibitors on IL-3-induced phosphorylation of CREB. TF-1 cells were cytokine-deprived for 18 h and restimulated with human IL-3 for the indicated times. Restimulation was performed in the absence or presence of a 30 μM concentration of H-7 or HA1004 and 5 μM GF109023X. TPA was used at 10 nM. Cell lysates were prepared and immunoblotted with equal numbers of cells loaded per lane. Immunoblots were probed with an Ab specific for CREB phosphorylated on Ser133 (A). Blots were subsequently stripped and reprobed with an Ab specifically recognizing CREB (B). Probed blots were developed by chemiluminescence and exposed to x-ray film.

when used as a contrasting pair at the appropriate concentrations, they can discriminate PKA from PKC (3). GF109023X (bisindolylmaleimide) specifically inhibits PKC in the micromolar range in cell culture without inhibiting PKA. Incubation of TF-1 cells with 5 μM GF109023X also completely abrogated IL-3-induced serine phosphorylation of CREB. The classic PKC activator 12-O-tetradecanoyl phorbol-13-acetate (TPA) also induced a marked increase in CREB phosphorylation in TF-1 cells. Both PKC inhibitors blocked TPA-induced CREB phosphorylation in TF-1 cells by phorbol ester at the appropriate concentrations (Fig. 1). Neither PKC inhibitor altered cell viability during the short time courses examined. We have shown previously that the time course for the induction of apoptosis with either cytokine withdrawal or PKC inhibition is 48 h (4, 6). Thus, we would not expect to see any apoptosis during the short time courses presented here.

NPC-15342, an inhibitor of the PKC regulatory domain, also blocks IL-3-induced CREB phosphorylation

Both H-7 and GF1090123X inhibit PKC by competing for ATP binding at the catalytic domain of the enzyme. To further ensure the specificity of our inhibition of CREB phosphorylation through PKC, we also tested the effect of a specific hexanamide derivative PKC inhibitor, NPC-15342, that binds to the regulatory domain of PKC (15). Very few protein kinases besides PKC have diacylglycerol binding domains, and NPC-15342 binding to this site lends great PKC specificity to this inhibitor. The k i for inhibition of PKC by NPC-15342 in intact cells is ~30 μM (15). Fig. 2 shows that between 5 and 100 μM NPC-15342 there was a concentration-dependent decrease in IL-3-induced phosphorylation of Ser133 CREB in factor-deprived TF-1 cells. CREB phosphorylation induced by phorbol ester (TPA) in TF-1 cells was also blocked by NPC-15342. Thus, two classes of structurally unrelated PKC inhibitors acting through two different sites on the enzyme block IL-3-induced CREB phosphorylation at the expected concentrations. These data strongly verify a role for PKC-mediated phosphorylation in the activation of this transcription factor after βcR engagement.

GM-CSF but not IL-4 induces PKC-dependent CREB phosphorylation in TF-1 cells

To determine whether other cytokines that use the βc subunit also stimulate a PKC-dependent phosphorylation of CREB, we analyzed the response of TF-1 cells to GM-CSF. The CREB Ser133 phosphorylation response to GM-CSF was maximal at 30 min after stimulation (Fig. 3). The relatively specific PKC inhibitor GF109023X also blocked GM-CSF-induced CREB Ser133 phosphorylation as did H-7. The PKA inhibitor HA1004 actually seemed to enhance GM-CSF-induced Ser133 phosphorylation. To determine whether cytokines that do not use the βc subunit also induce PKC-dependent CREB phosphorylation, we examined the level of CREB Ser133 phosphorylation in factor-deprived cells after the addition of IL-4. TF-1 cells express IL-4 receptors, and IL-4 initiates intracellular signaling in these cells (16). The IL-4R does not use the βc subunit but shares subunits with the IL-2R (16–18). Fig. 4 shows that the rapid phosphorylation of CREB Ser133 observed with IL-3 and GM-CSF was not observed with IL-4. Examination of later time points also did not show any increased levels of phosphorylation in response to IL-4 (data not shown). Therefore, it appears that PKC activation of CREB in myeloid cells may be limited to cytokines using the βcR subunit. Furthermore, work is necessary to extend these findings to other non-βc cytokines.

Does MAPK participate in a βc PKC-dependent CREB phosphorylation?

Previous work has shown that PKC is an upstream regulator of Raf-1 kinase, which is the kinase that activates MAP/extracellular signal-related kinase (ERK) kinase-1 (MAPKK), the upstream activator of p42 MAPK. If βc activation of PKC also activates MAPK, then we would expect that the cell-permeable specific

FIGURE 2. NPC-15342, an inhibitor of the PKC regulatory domain, blocks IL-3-induced CREB phosphorylation. TF-1 cells were cytokine-deprived for 18 h and restimulated with recombinant human IL-3 and GM-CSF was not observed with IL-4. Examination of later time points also did not show any increased levels of phosphorylation in response to IL-4 (data not shown). Therefore, it appears that PKC activation of CREB in myeloid cells may be limited to cytokines using the βcR subunit. Furthermore, work is necessary to extend these findings to other non-βc cytokines.
MAPK kinase inhibitor 2'-amino-3'-methoxyflavone, also known as PD 98059, would inhibit CREB phosphorylation in our system. This inhibitor blocks MAPK activation without inhibiting tyrosine kinases, PKC, or phosphatidylinositol 3-kinase. It has been used successfully to determine the role of MAPK in epidermal growth factor signal transduction and nerve growth factor-induced apoptosis (19, 20). We have found that 10 μM PD 98059 blocks GM-CSF-induced phosphorylation of CREB in TF-1 cells (Fig. 5). The inhibition was concentration-dependent between 1.0 and 10 μM (data not shown). The bisindolylmaleimide PKC inhibitor GO6908 completely inhibited IL-3-induced CREB phosphorylation, as observed in other experiments. Fig. 6 demonstrates that PKC was still activatable in TF-1 cells after inhibition of MAPK with PD 98059. Addition of phorbol ester to PD 98059-treated cells produced a strong activation of CREB phosphorylation as determined by immunoblotting, whereas the GM-CSF-induced CREB phosphorylation was blocked. All lanes were equally as determined by stripping the blot and reblotting with an Ab specific for CREB. The manufacturer’s specifications showed that the inhibitors of PKC used in these studies have no effect on MAPK phosphorylation activity. (data not shown). The activation of MAPK with these cytokines is independent of PKC.

PKC inhibitors block CRE-dependent transcription from the egr-1 promoter in myeloid cells

Previous work has shown that GM-CSF and IL-3 activate signaling pathways that result in rapid and transient activation of the egr-1 early response gene (14, 21). The transcriptional activation of this gene in response to the latter cytokines in TF-1 cells requires the presence of the CRE contained within the −116 nucleotide region of the egr-1 promoter. This region also contains an SRE, but no protection of the SRE in DNase I footprinting assays was observed. SRE/CAT constructs were not IL-3- or GM-CSF-responsive in TF-1 cells in transient transfection assays (21). The blocking of phosphorylated CREB to the CRE was shown to be essential for induction of a transcription response to GM-CSF or IL-3 (14, 21); however, the protein kinases responsible for the phosphorylation of the trans-activating CREB after βR engagement have not been determined. We used a set of previously described egr-1 promoter/CAT reporter constructions to confirm a role for PKC in CREB-dependent transcription in TF-1 cells. Three constructions were examined using transient transfection into TF-1 cells: 56, which only contains the TATA box promoter of the egr-1 gene; 116, which contains the promoter linked to a SRE and CRE; and 116 mutant in which the CRE has been deleted. TF-1 cells were deprived of cytokine for 12 h and then cotransfected by electroporation.
After stimulation for 4 h with cytokine, CAT activity was assayed. Transfection efficiency was corrected for in each transfection as previously described (6), and three individual transfections were performed for each condition in each independent experiment. Preliminary experiments using a β-galactosidase reporter driven by the CMV promoter indicated no difference in gene expression driven by the CMV promoter between control transfected cells and those incubated with PKA and PKC inhibitors for the 6 h required of the assays (data not shown). Table I shows that IL-3 and GM-CSF both stimulate the egr-1 enhancer promoter construction with increases in CAT expression of ~2-fold above unstimulated cells. This level of stimulation is similar to what has been reported previously with these cytokines using this construction (14, 21). IL-4, which neither uses the βR subunit nor stimulates CREB phosphorylation, does not drive this promoter construction. Fig. 7 shows that the 116 mutant construction lacking the CRE has neither basal expression in TF-1 cells nor activation of transcription by IL-3 (the 56 activity is background). The figure further shows that PKC inhibition by GF109023X blocks the ability of IL-3 to stimulate CAT expression from the 116 construction containing the CRE. Incubation of transiently transfected cells with the PKA inhibitor HA1004 does not inhibit IL-3 stimulation of CREB-dependent transcription from 116 in these cells. H-7 had the same effect as GF109023X (data not shown). Interestingly, GF109023X also inhibits the basal level of transcription from this construction; however, IL-3 is unable to restore the level of CAT expression to the basal unstimulated level in the presence of the inhibitor (Fig. 7, V6 116 + GF lane). We confirmed that the inhibition of CREB-dependent basal transcription is concentration-dependent for GF109023X between 1 and 5 μM (Table II). The inhibitors did not alter the viability of the transfecants after incubation. This demonstrates that PKC is required for βc cytokine stimulation as well as basal transcription from this CREB-dependent promoter.

Overexpression of PKC isoform ε but not δ enhances CREB-dependent transcription from the egr-1 promoter in myeloid cells

We have recently shown that TF-1 cells stably overexpressing the PKCe isoform are resistant to apoptosis after cytokine withdrawal and show enhanced expression of the bcl-2 survival gene (6). These findings coupled with the recent demonstration of a role for PKC-dependent phosphorylation of CREB in regulating bcl-2 expression in B cells led us to investigate the role of this novel PKC isoform in regulating transcription from the CRE containing 116 promoter/reporter construction. Vector, PKCe, and PKCδ stable TF-1 transfecants that we previously described were used as hosts for transient transfection experiments with the egr-1 promoter/CAT reporter constructions. Cells were deprived of cytokine overnight, and equal numbers of viable cells of each type were electroporated with the 116 construct and a control plasmid to monitor transcription efficiency. Six hours after transfection, cells were lysed, and lysates assayed for CAT activity. The 116 mutant construction (missing the CRE) did not signal in any of the vector or PKC-transfected cell lines described (data not shown). This result is similar to the lack of expression of this CRE-deleted construction observed in cytokine-stimulated wild-type cells (Fig. 7). Fig. 8 shows that after transfection of the 116 construction (containing the CRE) into the e1 and e10 cell lines, the basal level of CAT expression was reproducibly 2- to 2.5-fold greater than the basal activity in either the vector or PKCδ-transfected cell lines. In fact, it was observed that CAT expression was significantly inhibited in the PKCδ 25 transfecant. Although the responses of the transfected cell lines to IL-3 was somewhat reduced in these experiments compared with wild-type cells, the IL-3-stimulated levels in the PKCe-overexpressing cell lines were, nevertheless, higher than in any of the other transfected cell lines stimulated with cytokine and assayed for CAT expression.

PKC inhibitor GF109023X reduces CRE-dependent transcription in PKCe-overexpressing cell lines

It has been previously demonstrated that increased levels of PKC protein yield levels of enzyme activity comparable to or greater

Table I. Effects of cytokines on CREB-dependent transcription in wild-type TF-1 cells

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>CAT Activity (cpm × 10^4)</th>
</tr>
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<tbody>
<tr>
<td>Deprived</td>
<td>30.0 ± 2.8</td>
</tr>
<tr>
<td>+IL-3</td>
<td>57.8 ± 0.56*</td>
</tr>
<tr>
<td>+GM-CSF</td>
<td>71.0 ± 7.0*</td>
</tr>
<tr>
<td>+IL-4</td>
<td>25.5 ± 2.4</td>
</tr>
</tbody>
</table>

* TF-1 cells were transfected as described in Materials and Methods and then stimulated with the indicated cytokines at 10 ng/ml. Six hours after stimulation, cells were harvested and lysed for CAT assays. Transfections were performed in triplicate and CAT assays corrected for transfection efficiency. The results presented are the mean ± SEM from one experiment. *, p < 0.05 compared to deprived cells using Student’s t test. The experiment was repeated three times with similar results.

FIGURE 7. PKC inhibitor blocks transcription from the CRE in the egr-1 promoter in TF-1 cells. TF-1 cells were deprived of cytokine and transiently cotransfected by electroporation with egr-1 promoter/CAT reporter constructions. Transfected cells were subsequently stimulated with human rIL-3 alone or in the presence of 5 μM PKC inhibitor bisindolylmaleimide GF109023X (GF) or 30 μM PKA inhibitor HA1004 for 4 h, and cell lysates were prepared for CAT assays. All transfections were performed in triplicate, and the CAT activities are corrected for transfection efficiency. The mean value is presented and the SE was <10% of the mean. *, p < .05 compared with IL-3-deprived cells using Student’s t test. The experiment presented is representative of three that gave similar results.
ylation. In TF-1 cells, all the cytokine receptors using the \( \beta_c \) subunit (i.e., IL-3 and GM-CSF) and inducing Janus kinase (JAK)-2 phosphorylation also induce phosphorylation of CREB on Ser 133 through PKC. These cells also express biologically active IL-4R; however, the IL-4R uses neither the \( \beta_c \) subunit nor JAK-2 for signaling. Instead, it associates with the common \( \gamma \) chain of the IL-2R and induces JAK-1 and JAK-3 phosphorylation in target cells (16, 18). However, IL-4 did not induce CREB Ser\(^{133} \) phosphorylation in TF-1 cells. Thus, the activation of CREB phosphorylation through PKC may be part of a signal transduction cascade shared among \( \beta_cR \)-using cytokines, but this needs to be more extensively studied. Previous work on the GM-CSF induction of the egr-1 gene also demonstrated phosphorylation of CREB on Ser\(^{133} \), but the kinases involved were not identified (7).

These results are consistent with results on CREB Ser\(^{133} \) phosphorylation in B lymphocytes stimulated by cross-linking the surface IgG (22). In B cells, down-regulation of PKC by extended incubation with phorbol ester blocks IgG stimulation of CREB phosphorylation. Furthermore, CD40 ligand and LPS, which do not activate PKC in B cells, do not induce CREB phosphorylation. Using relatively specific PKC inhibitors, it was also shown that PKC but not PKA or CaMK are required for surface IgG-stimulated CREB phosphorylation in B cells (23). In T cells, TCR-mediated CREB phosphorylation also requires PKC and is independent of PKA and CaMK activity (24). Thus, both myeloid and lymphoid cells with receptors capable of inducing CREB phosphorylation use a PKC cascade.

To determine whether PKC activation was directly required for \( \beta_cR \) cytokine induction of CREB phosphorylation we used an egr-1-based promoter construction containing an SRE and a CRE and inhibited by the structurally unrelated PKC inhibitors H-7 and GF109023X. The PKA and CaMK inhibitor HA 1004 did not inhibit cytokine-induced transcription from the construct. Previous DNase I footprinting assays of the SRE in this construct has shown no protection, and SRE/CAT constructs did not confer GM-CSF or IL-3 responsiveness in transient transfection assays (14, 21). The binding of phosphorylated CREB to the CRE in the 116 construction was shown to be the essential activation signal for the induction of a transcription response to GM-CSF in experiments transfecting the promoter/reporter construct with a vector overexpressing CREB (14). Interestingly, we also observed a

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\begin{align*}
&\text{FIGURE 8. The activity of the CRE in the egr-1 promoter in TF-1 cells overexpressing novel PKC isoform. TF-1 cell lines stably overexpressing the PKC \( \delta \) (88 and 825), PKC\( \varepsilon \) isoform (e1 and e10), or stably transfected with the expression vector (vector 6) were cytokine-deprived and transiently transfected with the -116 egr-1 promoter/reporter construction. The transfected cells were either not stimulated or stimulated with 10 ng/ml human rIL-3, and 6 h after incubation at 37°C were lysed and assayed for CAT activity. Transfections were performed in triplicate, and CAT activities were corrected for transfection efficiency. The mean value is presented and the SEM was <10% of the mean. The (+) and (++) indicate that p < .05 compared with IL-3-deprived or IL-3-stimulated vector 6-transfected cells, respectively, using Student’s t test. The experiment presented is representative of three that gave similar results.}
\end{align*}
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\begin{align*}
&\text{FIGURE 9. The effect of PKC inhibitor GF109023X on CRE-depend-}
&\text{ent egr-1 promoter activity in the PKC e1-overexpressing cell line. PKC}\( \varepsilon \)
&\text{were cytokine-deprived and transfected with the egr-1 promoter/reporter}
&\text{constructions. Transfected cells were then unstimulated, stimulated with 10}
&\text{ng/ml IL-3, or 10 ng/ml IL-3 in the presence of protein kinase inhibitors,}
&\text{and incubated at 37°C. At the end of the incubation, cells were lysed for}
&\text{CAT assay. Transfections were performed in triplicate, and CAT activities}
&\text{were corrected for transfection efficiency. The mean activity is presented}
&\text{and the SEM was <10% of the mean. *, p < .05 compared with the}
&\text{corresponding noninhibitor-treated control using Student’s t test. The re-}
&\text{sults presented are representative of three experiments with similar results.}
\end{align*}
\]
basal level of CAT expression in TF-1 cells, and this background level was also reduced by preincubation with the PKC inhibitors. CREB is resident in the nucleus, and our data would indicate that even under unstimulated conditions PKC provides a low basal level of phosphorylation. The CREB Ser133 immunoblots did not even under unstimulated conditions PKC provides a low basal level of expression and this background level was also reduced by preincubation with the PKC inhibitors. It should be noted that it was previously reported that IL-3 did not use CREB to stimulate transcription from this promoter (14).

However, the previous studies were conducted by cotransfecting plasmids overexpressing CREB or a mutated CREB with the reporter promoter construct. The lack of stimulation in these experiments may have been due to the high levels of background expression from the control-mutated construction that obscured the IL-3 stimulation. The experiments reported here using the endogenous CREB levels do not have this complication, and we were consistently able to observe an IL-3 stimulation of transcription.

Our transient transfections of CRE reporter promoter constructs into stable PKCε-overexpressing cell lines confirmed a role for this PKC isoform in cytokine-induced CREB phosphorylation in myeloid cells, and leads us to suggest an important role for this novel isoform in CREB phosphorylation. The PKCε cells had far higher levels of basal CAT gene expression than either the vector-transfected cells or the PKCδ-overexpressing cells. The fact that one novel isoform (ε) but not another (δ) can specifically enhance CREB phosphorylation suggests an important role for this specific isoform in regulating the activity of this transcription factor. Little information is available on PKC isoform specificity for transcription factor phosphorylation, and it would be quite interesting to know whether this isoform were also responsible for CREB phosphorylation in response to surface IgG cross-linking in B lymphocytes (22, 23). Although MSK-1 (24) and pp90 rsk-2 (ribosomal S6 kinase) are possible CREB kinases (25, 26) during signal transduction, direct evidence that GM-CSF activates rsk-2 phosphorylation of CREB has been published (26). This kinase acts downstream of stimulation by the ERK/p38 MAPK cascade (27–29), and PKCε has been shown to activate the ERK-1/p38 MAPK cascade through phosphorylation of Raf-1 (30). Our experiments inhibiting MAPK kinase activity resulting in inhibition of IL-3-induced CREB phosphorylation implicates the MAPK cascade in the βcR-induced phosphorylation of CREB. Bitorff et al. (31) have shown that in TF-1 cells both IL-3 and GM-CSF activate MAPK activity. They showed that IL-3 and GM-CSF showed the same degree of activation and kinetics of activation in TF-1 cells. Therefore, it is likely that a PKC activation of the MAPK cascade followed by rsk-2 phosphorylation of CREB is involved in βcR signal transduction. The incomplete inhibition of CREB phosphorylation observed in our studies suggests that PKC itself may also directly contribute to the phosphorylation of CREB. Several pieces of evidence suggest that PKC can phosphorylate CREB directly. The Ser117 in the CREB analog CRE modulator (CRE) that corresponds to 133 in CREB itself is phosphorylated directly by PKC or RSK (32–33). It is also established experimentally in vitro that the PKCε isoform can directly phosphorylate CREB (30). Investigation of the contribution of other PKC isoforms to the βc cytokine receptor-induced phosphorylation of CREB should be undertaken. Taken together, our data would suggest that IL-3/GM-CSF receptor engagement initiates a novel signal transduction pathway in which receptor-associated tyrosine kinases activate a PC-PLC, generating a sustained increase in diacylglycerol levels. This is followed by stimulation of PKCε, and PKCε induces phosphorylation of CREB through a combination of MAPK cascade activation of RSK-2 kinase and possible direct phosphorylation by PKC. The diagram in Fig. 10 presents a schematic of such a pathway.

Such a novel pathway has not received significant attention in studies of βcR cytokine signal transduction; however, recent data on the regulation of bcl-2 expression and apoptotic suppression emphasize the important functional role of such a signal transduction cascade. The activation of a PC-PLC and PKCε probably plays an important role in the regulation of genes containing an egr-1-like promoter containing a CRE. Expression of bcl-2 and perhaps other survival genes in response to IL-3 and GM-CSF is likely regulated by this cascade (6–10). In PKCε-overexpressing TF-1 cells, the high levels of bcl-2 expression can be reduced by PKC inhibitors. Overexpression of PKCε in TF-1 cells enhances the survival of these cells in the absence of cytokine and induces high level sustained bcl-2 gene expression (10). Studies of the bcl-2 promoter in B cells have revealed that this promoter contains a positive regulatory region containing a CRE element (11). Crosslinking of surface Ig receptor on mature B cells leads to increased bcl-2 promoter activity through a PKC-dependent pathway involving phosphorylation of CREB. Reagents that increased cAMP levels had no effect on the transcription from the CRE-containing promoter element in these cells. Furthermore, a similar PKC-dependent phosphorylation of CREB was involved in the enhanced bcl-2 expression observed in immature B cells treated with survival signals like phorbol ester (11). It would thus appear likely
that in βc-dependent meyloid cell lines regulation of bcl-2 gene expression occurs through the activation of PKCe and subsequent phosphorylation of CREB Ser133 leading to CREB/CBP binding to the CRE in the bcl-2 downstream regulatory region. A definite role for CREB in regulating bcl-2 expression will require appropriate experiments with a bcl-2 reporter promoter construction.

A role for CREB phosphorylation in the suppression of apoptosis has also been shown in human melanoma cells (34). A dominant negative form of CREB transfected into human melanoma cells rendered them susceptible to apoptosis induced by increasing intracellular calcium ion concentrations. In addition, this dominant negative form also inhibited CAT expression from a CRE-dependent signal transduction elements as well as receptor components in TF-1 cells. Thus, CREB phosphorylation may be important in the regulation of expression of genes contributing to cell survival and suppression of apoptosis in a number of different cellular systems.

References