Molecular Biology of the Cell
Vol. 13, 4497–4507, December 2002

Calcium Regulation of GM-CSF by Calmodulin-Dependent Kinase II Phosphorylation of Ets1

Hebin Liu and Thomas Grundström*

Department of Molecular Biology, Umeå University, S-901 87 Umeå, Sweden

Submitted March 18, 2002; Revised August 15, 2002; Accepted September 4, 2002
Monitoring Editor: Carl-Henrik Heldin

The multipotent cytokine granulocyte macrophage-colony stimulating factor (GM-CSF) is involved in particular in the physiological response to infection and in inflammatory responses. GM-CSF is produced by many cell types, including T lymphocytes responding to T-cell receptor activation and mantle zone B lymphocytes. B-cell receptor and T-cell receptor activation generates two major signals: an increase in intracellular Ca^{2+} concentration and a protein kinase cascade. Previous studies have shown that the Ca^{2+}/calmodulin-dependent phosphatase calcineurin mediates stimulation of GM-CSF transcription in response to Ca^{2+}. In this study, we show that Ca^{2+} signaling also regulates GM-CSF transcription negatively through Ca^{2+}/calmodulin-dependent kinase II (CaMK II) phosphorylation of serines in the autoinhibitory domain for DNA binding of the transcription factor Ets1. Wild-type Ets1 negatively affects GM-CSF transcription on Ca^{2+} stimulation in the presence of cyclosporin A, which inhibits calcineurin. Conversely, Ets1 with mutated CaMK II target serines showed an increase in transactivation of the GM-CSF promoter/enhancer. Moreover, constitutively active CaMK II inhibited transactivation of GM-CSF by wild-type Ets1 but not by Ets1 with mutated CaMK II sites. Mutation of CaMK II target serines in Ets1 also relieves inhibition of cooperative transactivation of GM-CSF with the Runx1/AML1 transcription factor. In addition, the Ca^{2+}-dependent phosphorylation of Ets1 reduces the binding of Ets1 to the GM-CSF promoter in vivo.

INTRODUCTION

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multipotent cytokine involved in the production and function of hematopoietic cells. In particular, GM-CSF plays a major role in the physiological response to infection and in inflammatory responses. GM-CSF and related cytokines can augment the functional antimicrobial activities of macrophages, monocytes, and neutrophils (Liles, 2001). A broad range of cells, including T cells of both the Th1 and Th2 phenotype, mantle zone B lymphocytes, macrophages, mast cells, endothelial cells, fibroblasts, and epithelial cells, are all capable of GM-CSF production in response to different immune-activating and inflammatory stimuli (Gasson, 1991; Pistoia and Corcione, 1995). Moreover, GM-CSF production can be constitutive in certain mature B-cell acute lymphoblastic leukemia cell lines (Estrov *et al.*, 1996–1998). T cells responding to T-cell receptor (TCR) activation is a major source of GM-CSF. TCR activation generates two major signals: an increase in intracellular calcium that can be mimicked by treatment with a Ca^{2+} ionophore and a protein kinase cascade that can be mimicked by treatment with a protein kinase C–activating phorbol ester. Changes at the transcriptional level are important for the control of GM-CSF expression. The GM-CSF promoter region is highly conserved between the mouse and human genes. Both genes also contain similar powerful enhancers that are located ~2–3 kb upstream from the transcription start. The promoter and the enhancer are both involved in the response of GM-CSF to TCR activation. A number of conserved *cis*-acting elements identified in the promoter are important for its activity. These elements include the conserved lymphokine element 0 (CLE0), which contains binding sites for Ets and AP-1 transcription factors and is located ~40 base pairs (bp) upstream from the transcription start. Nearby on the upstream side is a binding site for Runx1 (also denoted AML1), and farther upstream are binding sites for Sp1 and nuclear factor (NF)-κB located ~70–90 bp from the start site. Stimulation of GM-CSF transcription by Ca^{2+} is mediated by the calmodulin-dependent phosphatase calcineurin, and the transcription factors NF-AT, AP-1, and NF-κB have been implicated in this activation (for review and references, see Shannon *et al.*, 1997; see also Shang *et al.*, 1999).

Ets1 is the founding member of the Ets family of transcription factors. It plays an important role in regulation of critical genes involved in cell proliferation, differentiation, de-

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E02–03–0149. Article and publication date are at www.molbiolcell.org/cgi/doi/10.1091/mbc.E02–03–0149.

* Corresponding author. E-mail address: Thomas.Grundstrom@molbiol.umu.se.

velopment, transformation, angiogenesis, and apoptosis. Ets1 is highly expressed in cells of the T and B lymphoid lineages and is important for their normal differentiation, homeostasis, and activation (Bories *et al.*, 1995; Muthusamy *et al.*, 1995). Regulation of the GM-CSF promoter by Ets1 has been studied extensively. In addition to the Ets1 binding site in the CLE0 element, weaker Ets1 sites are also observed farther upstream in the promoter. Furthermore, Ets1 can transactivate the human GM-CSF promoter in Jurkat T cells stimulated with the Ca^{2+} ionophore ionomycin and the phorbol ester phorbol 12-myristate 13-acetate (PMA) (Thomas *et al.*, 1995). Ets1 has also been reported to upregulate GM-CSF expression in mast cells (McKinlay *et al.*, 1998). Ets1 stimulates the GM-CSF promoter in a synergistic relationship with NF-κB and AP-1 (Shannon *et al.*, 1997; Thomas *et al.*, 1997).

Ets1 becomes rapidly phosphorylated on antigenic stimulation of T or B lymphocytes or on treatment with ionomycin. These phosphorylations are transient and dependent on the increase in intracellular calcium concentration (Pognonec *et al.*, 1990; Fisher *et al.*, 1991; Rabault and Ghysdael, 1994). A major site of Ca^{2+}-dependent phosphorylation of Ets1 is a serine cluster in exon VII adjacent to the Ets DNA binding domain (Fisher *et al.*, 1994; Rabault and Ghysdael, 1994; Cowley and Graves, 2000). Ets1 has been identified as a calmodulin-dependent kinase II (CaMK II) target in vitro (Fisher *et al.*, 1994). Moreover, phosphorylation of the serines adjacent to the DNA binding domain by CaMK II can specifically inhibit the DNA binding of Ets1 in vitro through a mechanism of enforcing and stabilizing an autoinhibitory conformation (Cowley and Graves, 2000). However, it is not known whether Ca^{2+}-dependent phosphorylation of Ets1 affects the transcription of any gene in vivo.

In this study, we show that Ca^{2+} signaling not only positively regulates transcription from the GM-CSF promoter/enhancer through calcineurin but also negatively regulates it through CaMK II phosphorylation of serines in the autoinhibitory domain for DNA binding of Ets1. Moreover, this Ca^{2+}-dependent phosphorylation of Ets1 reduces the DNA binding of Ets1 to the GM-CSF promoter in vivo.

MATERIALS AND METHODS

Expression and Reporter Plasmids

The wild-type human CaMK $II_{\gamma\beta}$ eukaryotic expression plasmid, the inactive T286A and constitutively active T286D derivatives, and the parental expression plasmid pSRα.BKS have been described previously (Nghiem *et al.*, 1993). The Runx1 expression plasmid pBJ9AML1b has also been described previously (Xie *et al.*, 1999). The pCDNA-hEts1 plasmid encoding full-length human Ets1 cDNA was a kind gift from Dr. Sven Pettersson, Karolinska Institute. The *Escherichia coli* Ets1 expression plasmid pET20bEts1-wt was constructed by PCR with Pfu^{Turbo} DNA polymerase (Stratagene, La Jolla, CA). *Nde*I and *Hin*dIII sites were incorporated at the ends of the cDNA using the upstream primer 5′-GGGAATTC*CATATG*AAGGCGGCCGTCGAT-3′ and the downstream primer 5′-CCC*AAGCTT*CTCGTCGGCATCTGGCTTG-3′. The amplified DNA fragment was subcloned with *Nde*I and *Hin*dIII into the *E. coli* expression plasmid pET20b+ (Novagen, Madison, WI).

The eukaryotic Ets1 expression plasmid pBJ9Ets1-wt was constructed by subcloning the Ets1 cDNA into the pBJ9Ω vector, a kind gift from Dr. H. Land, with *Hin*dIII and *Bgl*II. These sites were introduced at the ends of the Ets1 cDNA by PCR with Pfu^{Turbo} DNA polymerase by use of the primers 5′-CCC*AAGCTT*CATATGAAGGCGGCCGTCGAT-3′ and 5′-GGA*AGATCT*TCACTCGTCGGCATCTGG-3′.

The mutants were obtained by PCR-based mutagenesis with overlapping DNA segments by use of the Pfu^{Turbo} DNA polymerase and the same external primers as above. The complementary internal primers for the serine 251 and 257 mutation to alanine were 5′-AC***G***CTTTTGAAAGCATAGAG***GC***CTACGATAGTTGTG-3′ and 5′-AG***GC***CTCTATGCTTTCAAAAG***C***GTCCTGGCCCCGAG-3′, and for the serine 282 and 285 mutation to alanine, 5′-GTTCCC***G***C-CTATGA***TGCA***TTCGACTCAGAGGACTATCC-3′ and 5′-GAGTC-GAA***TGCA***TCATAGG***C***GGGAACACGCTGCAGGC-3′ (mutations are in boldface/italics). The complementary internal primers for the C-terminal deletion mutant ΔEts1 (amino acids 1–315) were 5′-ACCGTGCTGACCTCAAT***T***AGGACAAGCCTGTCATTCC-3′ and 5′-GGAATGACAGGCTTGTCCT***A***ATTGAGGTCAGCACGGT-3′. The full-length Ets1 mutants were cloned into the *Nde*I/*Hin*dIII-digested pET20b+ *E. coli* expression vector and into the *Hin*dIII/*Bgl*II-digested pBJ9Ω eukaryotic expression plasmid. All PCR-generated DNA sequences were confirmed by DNA sequencing.

The enhancer and promoter of GM-CSF (Cockerill *et al.*, 1993) were obtained from genomic Jurkat DNA by PCR amplification with Pfu polymerase and cloned into the *Bgl*II and *Hin*dIII sites, respectively, of the pGL2-Basic reporter plasmid (Promega, Madison, WI). Existing *Bgl*II and *Hin*dIII sites of the 716-bp enhancer and the 0.6-kb promoter segment, respectively, were used, except at the 3′ end of the promoter, where the *Hin*dIII site was created with the 5′-GAGAAGCTTTAGCCTTTCTCTCTGTG-3′ primer. The *Hpa*I/*Sma*I segment of pGL2-Basic (nucleotides 5451–5453) was deleted to remove a potential Runx1 site. Sequencing of the reporter plasmid showed no differences compared with the previously isolated enhancer and promoter in any part reported to be important for transcription or containing any protein-binding site (Cockerill *et al.*, 1993). Compared with the reported isolates, an extra G was found at position 108 in the enhancer, and the nucleotides TC were absent at position 179–180 of the promoter.

Expression in E. coli *and Purification of Ets1 Proteins*

Ets1 variants were expressed from the plasmids pET20bEts1-wt and pET20bEts1-m3 in *E. coli* BL21(DE3) (Stratagene) as a fusion protein with a C-terminal His_6 tag according to the manufacturer's instructions. Harvested cells were lysed by freeze-thawing followed by sonication and centrifugation. Supernatants were mixed with Ni-NTA agarose (Qiagen, Hilden, Germany) at 4°C for 1 h and washed according to the manufacturer's instructions. The proteins were eluted by increasing the imidazole concentration to 250 mM. The Ets1-containing fractions were further purified by HiPrep 16/10 DEAE ion-exchange chromatography using the UNICORN fast protein liquid chromatography system (Amersham Biosciences, Arlington Heights, IL). Purified Ets1 proteins were dialyzed against 25 mM Tris-HCl, pH 7.5, 5% glycerol, 10 mM NaCl, 0.1 mM EDTA at 4°C. The concentrations of proteins were determined with the BCA protein assay kit (Pierce), and protein aliquots were stored at −80°C.

Cell Lines and Transient Transfections

The human malignant cell lines DG75, an Epstein-Barr virus–negative Burkitt's lymphoma; Jurkat, a human T-cell line; Raji, a human Epstein-Barr virus–positive Burkitt's lymphoma; and K562, an early erythroleukemia cell line were cultured as previously described (Lars and Paschalis, 1993; Hughes *et al.*, 1998). Transient transfections were performed as previously described (Lars and Paschalis, 1993) with 2 μg hCMV-β-gal plasmid (reference plasmid for normalization), 4 μg reporter plasmid, and 5 μg of each expression plasmid indicated. Where necessary, the corresponding empty expression vector was added to a total of 10 μg expression plasmids. Cells (1×10^7) were electroporated, followed by incubation in 10 ml

of medium. Where indicated, the culture was divided 30 min after electroporation, and 5 ml of the cells were stimulated with ionomycin (1 μg/ml, Calbiochem, La Jolla, CA), PMA (25 μg/ml, Sigma, St. Louis, MO), and/or cyclosporin A (CsA) (20 nM, Sigma) (final concentrations), and 5 ml was kept unstimulated. The cells were harvested after 20 h incubation.

CaMK II Phosphorylation of Ets1 In Vitro

Ets1 was in vitro phosphorylated with baculovirus-produced monomers of the α-subunit of CaMK II (New England Biolabs, Beverly, MA). CaMK II phosphorylations were performed at 30°C for 1.5 h in kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA) with 2.4 μM calmodulin, 2 mM $CaCl_2$, 10 μg purified His-tagged Ets1 wild-type or mutant protein, 200 μM γ-[^{32}P]-ATP, and the indicated amount of kinase. Reactions were stopped in SDS-PAGE sample buffer by boiling for 5 min and then analyzed by 10% SDS-PAGE. Gels were stained with Coomassie blue, destained, dried, and exposed to autoradiography film overnight. The level of phosphorylation was analyzed with an Image Quant phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was performed as previously described (Orlando *et al.*, 1997) with a few modifications. Briefly, 1×10^7 DG75 cells were cotransfected with 5 μg GM-CSF promoter/enhancer reporter and 5 μg pBJ9Ets1-wt or pBJ9Ets1-m3 with or without 5 μg constitutively active CaMK II. Where indicated, the transfected cells were stimulated with ionomycin in the presence of CsA 30 min after electroporation. Ten hours after transfection, the cells were cross-linked with formaldehyde (final concentration 1%, vol/vol) in RPMI medium at 4°C for 1 h, followed by the addition of glycine to a final concentration of 125 mM to inhibit further cross-linking. Cells were harvested by centrifugation and washed twice for 15 min with PBS at room temperature. The cells were lysed in RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with the complete EDTA-free protease inhibitor cocktail (Roche) and sonicated to solubilize the chromatin. The cell lysates were precleared by incubation with 30 μl protein G-Sepharose beads (Amersham Biosciences) followed by centrifugation. The supernatants were then incubated with anti-E12 antibody or C-275 anti-Ets1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. DNA–protein complexes were collected with protein G-Sepharose followed by washing 3 times with RIPA buffer. Bound DNA–protein complexes were treated with proteinase K at 56°C for 1 h and eluted from the antibodies with two incubations in 10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.5% SDS at room temperature for 30 min. Samples were then extracted twice with phenol/chloroform and precipitated with ethanol in presence of glycogen carrier. DNA fragments were recovered by centrifugation, resuspended in H_2O, and used for PCR amplifications. The PCR products were fractionated on 2.0% agarose gels and stained with ethidium bromide. The primers for ChIP flanking four Ets1 binding sites and spacing 285 bp in the GM-CSF promoter were 5′-CCCATTCAGACTGCCCAG-3′ and 5′-TCTGTGTAGCTGGGCTCACTG-3′.

RESULTS

Calcium Signaling Regulates the GM-CSF Promoter/Enhancer Positively through Calcineurin and Negatively by a Calcineurin-Independent Mechanism

To analyze the effects of an increased intracellular calcium ion concentration on GM-CSF transcription, we used a reporter plasmid containing the human GM-CSF promoter and enhancer. The Ca^{2+} ionophore ionomycin was used to create changes in the intracellular Ca^{2+} concentration in the presence and absence of the phorbol ester PMA. The effect of Ca^{2+} was analyzed both in the Jurkat T helper cell line and in the B-lymphocyte cell line DG75. In both cell lines, ionomycin in combination with the phorbol ester showed a strong positive effect on the GM-CSF promoter/enhancer (Figure 1). However, the GM-CSF activation was negligible in the presence of CsA, a drug that inhibits calcineurin. This is consistent with previous reports that GM-CSF activation by ionomycin in the presence of phorbol ester is calcineurin dependent (Shannon *et al.*, 1997). It is also noteworthy that ionomycin alone had a small but significant positive effect in both cell lines (Figure 1). Interestingly, the calcineurin inhibitor CsA not only blocked this activation but also decreased the GM-CSF activity slightly below the level of nontreated Jurkat cells and to almost a 2.5-fold lower level than the

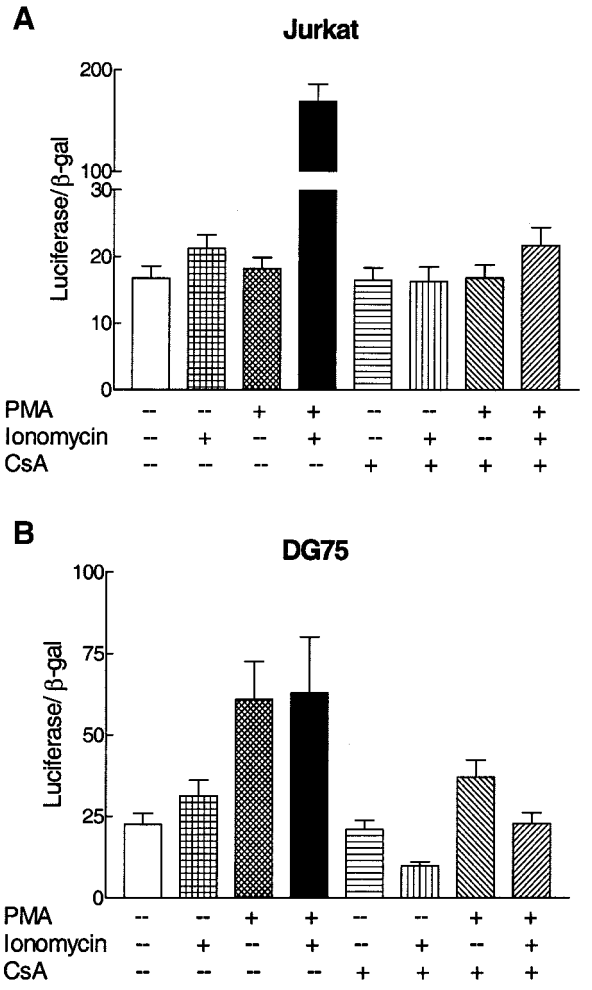

Figure 1. Effects of the phorbol ester PMA, the Ca^{2+} ionophore ionomycin, and the calcineurin inhibitor CsA on the GM-CSF promoter/enhancer in (A) the T-helper cell line Jurkat and (B) the B-lymphocyte cell line DG75. Bars represent average ratio of luciferase/β-galactosidase activity in arbitrary units from three independent transfections ± SD, using β-galactosidase expression from an hCMV-β-gal plasmid for normalization.

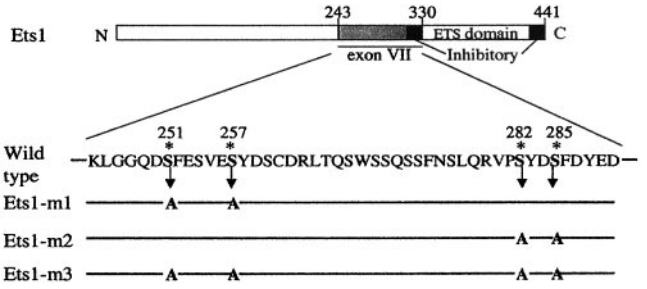

Figure 2. Schematic of the structure of the human Ets1 protein. The positions of the CaMK II target serines in exon VII are indicated by an asterisk. Three mutants were constructed in which different combinations of these serines were replaced with alanine. Amino acid sequence is shown in single-letter code.

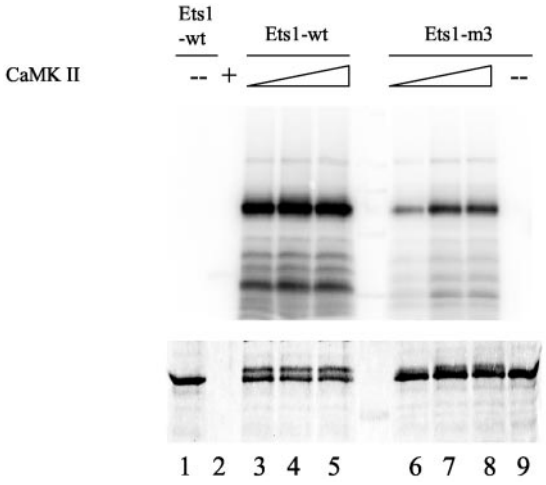

Figure 3. Phosphorylation of Ets1 wild-type and Ets1-m3 mutant by CaMK II in vitro. Ets1 proteins were separated by 10% SDS-PAGE, and the phosphorylated form was detected by autoradiography (top), whereas both forms were detected by Coomassie blue staining (bottom). The amounts of baculovirus-produced CaMK II (New England Biolabs) used were 125 U in lanes 3 and 6, 250 U in lanes 2, 4, and 7, and 500 U in lanes 5 and 8.

nontreated DG75 cells. Thus, we conclude that in addition to the established positive calcineurin-dependent effect of Ca^{2+} on GM-CSF expression, there is also a negative Ca^{2+} signaling effect on GM-CSF that appears to be calcineurin independent. PMA had a strong activating effect in DG75 cells without ionomycin as well. To a large extent, this activation was inhibited by CsA, suggesting that the intracellular calcium ion concentration in the presence of PMA was sufficient to activate calcineurin in this cell line. This finding is not surprising, considering that a calcineurin-activated transcription factor, NF-AT, which participates in regulation of GM-CSF (Shannon *et al.*, 1997; Feske *et al.*, 2000), can react on smaller Ca^{2+} concentration increase than other Ca^{2+}-activated transcription factors (Dolmetsch *et al.*, 1997). Importantly, in this cell line, a further increase in Ca^{2+} concentration by ionomycin treatment in the presence of PMA and CsA led to a smaller GM-CSF activation than without ionomycin. Thus, Ca^{2+} signaling can also have a negative calcineurin-independent effect on GM-CSF in the presence of phorbol ester.

Increased Transactivation of the GM-CSF Promoter/Enhancer by Ets1 with Mutated CaMK II Sites

The Ets1 transcription factor makes a large contribution to the activity of the GM-CSF promoter/enhancer (Thomas *et al.*, 1995; Shannon *et al.*, 1997; Thomas *et al.*, 1997; McKinlay *et al.*, 1998) and is therefore a putative target for a calcium ion effect on GM-CSF transcription. Four internal serines, located amino-terminal to the Ets domain, are Ca^{2+}-dependent phosphorylation targets (Rabault and Ghysdael, 1994; Cowley and Graves, 2000). Significantly, phosphorylation of Ets1 by Ca^{2+}-dependent pathways is thought to inhibit DNA binding in vitro (Fisher *et al.*, 1994; Rabault and Ghysdael, 1994; Cowley and Graves, 2000). To analyze the role of these four serines, S251, S257, S282, and S285, in transcription, we constructed three mutant derivatives of human Ets1 (Figure 2). The mutant Ets1-m1 contains S251A and S257A substitutions, Ets1-m2 contains S282A and S285A substitutions, and Ets1-m3 contains all these four substitutions. Treatment of Ets1 by T-cell nuclear extract or phosphorylation of these four serines by calmodulin-dependent kinase II (CaMK II) has recently been reported to decrease Ets1 DNA binding by reinforcing autoinhibition (Cowley and Graves, 2000). To confirm that these sites can be specifically and efficiently phosphorylated by CaMK II, His-tagged wild-type and Ets1-m3 proteins were produced in *E. coli*, purified, and used as a substrate for CaMK II in vitro (Figure 3). Wild-type Ets1 was efficiently phosphorylated by CaMK II, as evidenced by ^{32}P incorporation and by a retarded band after separation by PAGE and staining of the gel with Coomassie blue. In contrast, the level of phosphorylation was dramatically decreased in the Ets1-m3 mutant, with all four reported CaMK II phosphorylation sites replaced with alanine, and very little of the protein had altered mobility (Figure 3). A higher concentration of CaMK II was needed to reach maximal phosphorylation of Ets1-m3, and this level was still 5.1-fold lower than for the wild type. This is in agreement with a recent report indicating that mutation of these four serines reduced CaMK II phosphorylation from approximately 5 to 1 mole of phosphate per mole of Ets1 (Cowley and Graves, 2000). These findings confirm that Ets1 can be efficiently and specifically phosphorylated by CaMK II in vitro and that the four mutated serines are major phosphorylation sites.

Ets1 transactivates the GM-CSF promoter in a PMA- and ionomycin-dependent manner in Jurkat T cells (Thomas *et al.*, 1995). To analyze whether Ets1 has the corresponding effect on regulation of GM-CSF expression in DG75 cells, we used the GM-CSF promoter/enhancer reporter construct along with expression vectors for wild-type or mutant Ets1 in transient transfections of DG75 cells with or without subsequent stimulation with PMA and ionomycin (Figure 4A). In the absence of stimulation, expression of wild-type Ets1 had no positive effect on the expression of the reporter. In contrast, expression of Ets1-m1 or Ets1-m2, each with two of the phosphorylation sites replaced by alanine, increased expression of the GM-CSF reporter 1.7- and 1.5-fold, respectively. Moreover, Ets1-m3 with all four phosphorylation

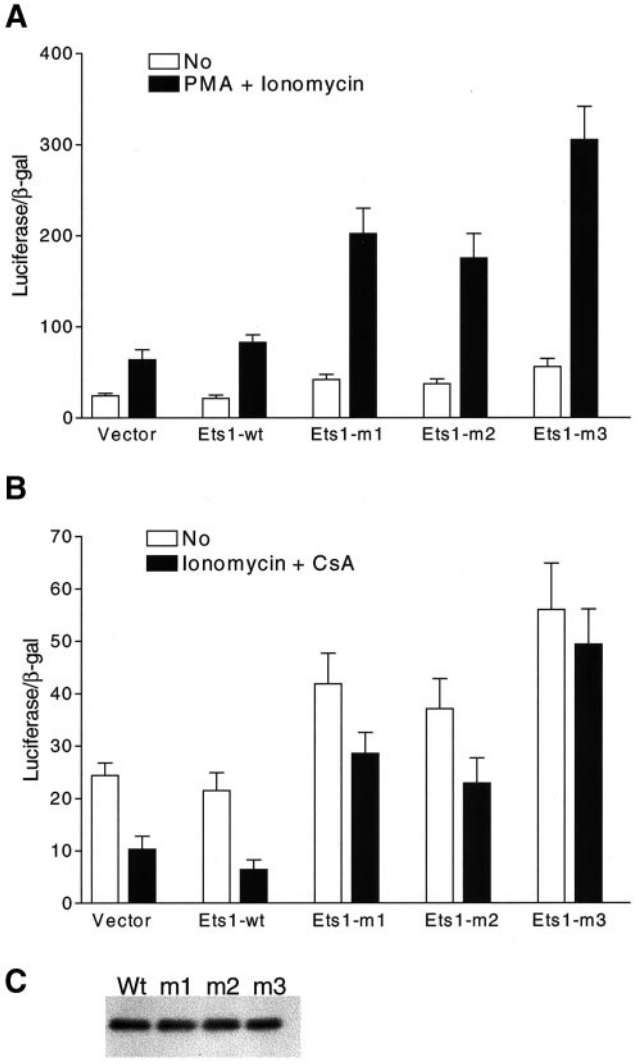

Figure 4. Effects of Ets1 wild-type and mutants on the GM-CSF promoter/enhancer in DG75 cells in the absence and presence of the phorbol ester PMA, the Ca^{2+} ionophore ionomycin, and the calcineurin inhibitor CsA. (A) Effects of treatment with PMA plus ionomycin and (B) effects of treatment with ionomycin plus CsA. Bars represent average ratio of luciferase/β-galactosidase activity in arbitrary units from three independent transfections ± SD, using β-galactosidase expression from an hCMV-β-gal plasmid for normalization. (C) Quantification of the expression level of Ets1 wild-type and the mutants in the transfected DG75 cells by Western blot using the C-275 anti-Ets1 antibody (Santa Cruz).

sites replaced by alanine could transactivate the reporter construct by 2.4-fold. In addition, each Ets1 mutant increased transactivation of the GM-CSF reporter considerably more than the wild type when the cells were treated with PMA and ionomycin. Expression of Ets1-m3 increased activation of the GM-CSF reporter 11.3-fold on treatment with PMA and ionomycin, whereas wild-type Ets1 increased activation only 3.1-fold (Figure 4A). The mutants Ets1-m1 and Ets1-m2 had intermediate effects (Figure 4A). The positive effects of the Ets1 mutations were not through increased expression of the mutated proteins, because Western blot analysis of transfected cells showed equal expression of transfected Ets1 wild-type and mutant constructs (Figure 4C). Furthermore, the increase is not a result of a changed nuclear distribution of Ets1, because mutations in the CaMK II sites of Ets1 do not affect its nuclear translocation (Rabault and Ghysdael, 1994). Taken together, these data show that mutations in the CaMK II sites of Ets1 increase transactivation of the GM-CSF reporter in DG 75 cells regardless of PMA and ionomycin stimulation.

Ets1 Negatively Affects GM-CSF Transcription on Stimulation with Ionomycin in the Presence of CsA

To examine the role of calcium-dependent Ets1 phosphorylation on transcriptional activation of GM-CSF without interference of calcineurin activation, the transfected cells were treated with ionomycin in the presence of CsA. This Ca^{2+} increase, without calcineurin activation, led to a 3.8-fold inhibition of transactivation of the reporter in the presence of wild-type Ets1 (Figure 4B). In contrast, the negative effect was greatly reduced for all mutants, illustrated by a minimal inhibition of only 1.2-fold for the combined mutant, Ets1-m3 (Figure 4B). These results indicate that the activity of Ets1 is sensitive to activation of one or more calcium ion–dependent enzyme(s) other than calcineurin. Conversely, Ets1 with mutations in some or all of the CaMK II target serines progressively loses this sensitivity. We conclude that the serines in Ets1 identified as CaMK II phosphorylation sites in vitro play a role for negative calcium ion–dependent regulation of the GM-CSF promoter/enhancer in vivo.

CaMK II Target Serines in Ets1 Inhibit the Cooperative Transactivation of GM-CSF by Runx1/AML1

The GM-CSF promoter contains a binding site for proteins belonging to the Runx family of transcription factors. The site is only a few nucleotides from the Ets1 site. One Runx family member is Runx1, which is essential for development of hematopoietic stem cells and several functions in hematopoiesis and the immune system (Lutterbach and Hiebert, 2000; Tracey and Speck, 2000). Cooperation between Ets1 and Runx1 has been reported in the transcription of several promoters, and Runx1 has been shown to decrease autoinhibition of Ets1 DNA binding through interaction between the proteins (Kim *et al.*, 1999; Goetz *et al.*, 2000). To analyze whether the negative calcium ion–dependent regulation of GM-CSF was blocked by Runx1, the largest splice form of mouse Runx1 was overexpressed. As expected, a functional cooperation was found between Ets1 and Runx1 in regulation of transcription of GM-CSF. Although Ets1 overexpression had a slightly negative effect (Figure 4) and Runx1 a small positive effect (Figure 5), the effect of combined overexpression of both Ets1 and Runx1 was a profound 5.6-fold activation (Figure 5). Importantly, the negative effect of ionomycin plus CsA was not blocked when Ets1 was cooperating efficiently with Runx1, because a 5.1-fold inhibition was observed (Figure 5), compared with 3.8-fold inhibition without Runx1 coexpression (Figure 4). Furthermore, all three Ets1 mutants decreased the negative effect of ionomycin plus CsA to approximately the same extent in the presence of

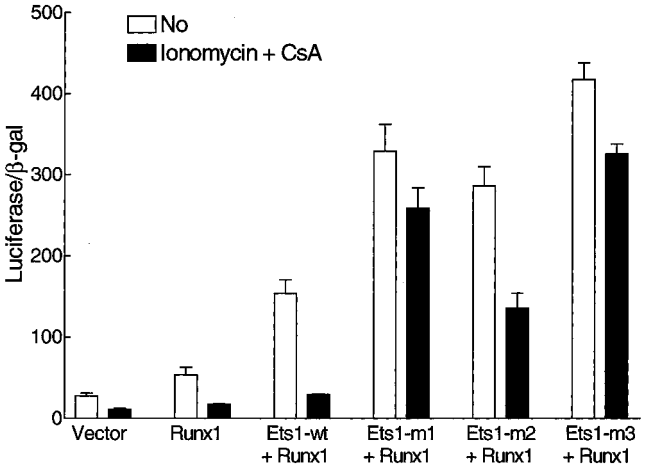

Figure 5. Effects of ionomycin plus CsA on activation of the GM-CSF promoter/enhancer by Ets1 wild type and mutants when the largest splice form of mouse Runx1 is overexpressed in DG75 cells. Assay conditions were as described in the legend to Figure 4.

strong Runx1 cooperativity as in the absence of Runx1 overexpression. The high calcium ion concentration in the absence of calcineurin activity gave as much as 29.8-fold higher GM-CSF reporter transcription on overexpression of Ets1-m3 together with Runx1 compared with the vector control. These results show that the serines in Ets1 identified as CaMK II phosphorylation sites in vitro play a significant negative role in calcium ion regulation of the GM-CSF promoter/enhancer even when Ets1 is efficiently cooperating with Runx1.

Constitutively Active CaMK II Inhibits Ets1 Transactivation of GM-CSF

To further investigate the negative effect of Ca^{2+} on Ets1 transactivation of GM-CSF, we coexpressed the wild-type or mutant Ets1 proteins with inactive or constitutively active CaMK II in the DG 75 cells. As shown in Figure 6A, expression of constitutively active CaMK II dramatically decreased transactivation of GM-CSF by Ets1. The effect of CaMK II was dependent on the kinase activity of the enzyme, because no significant effect was obtained by expression of inactive CaMK II. The background GM-CSF reporter transcription in the absence of Ets1 overexpression was also inhibited by constitutively active CaMK II, suggesting that this transcription is dependent on endogenous Ets1 and/or an equivalent CaMK II–sensitive protein. Similar to the effect of ionomycin in the presence of the calcineurin inhibitor CsA, the inhibition by constitutively active CaMK II was greatly alleviated by the mutations of the CaMK II target sites in Ets1. Only a very small part of the negative effect of constitutively active CaMK II remained in the Ets1-m3 mutant with all four identified CaMK II sites in Ets1 mutated. Thus, the negative effect of CaMK II overexpression on GM-CSF transcription by Ets1 is dependent on the serines that are CaMK II phosphorylation sites.

To analyze whether CaMK II can affect cooperation between Ets1 and Runx1, we also analyzed the effect of con-

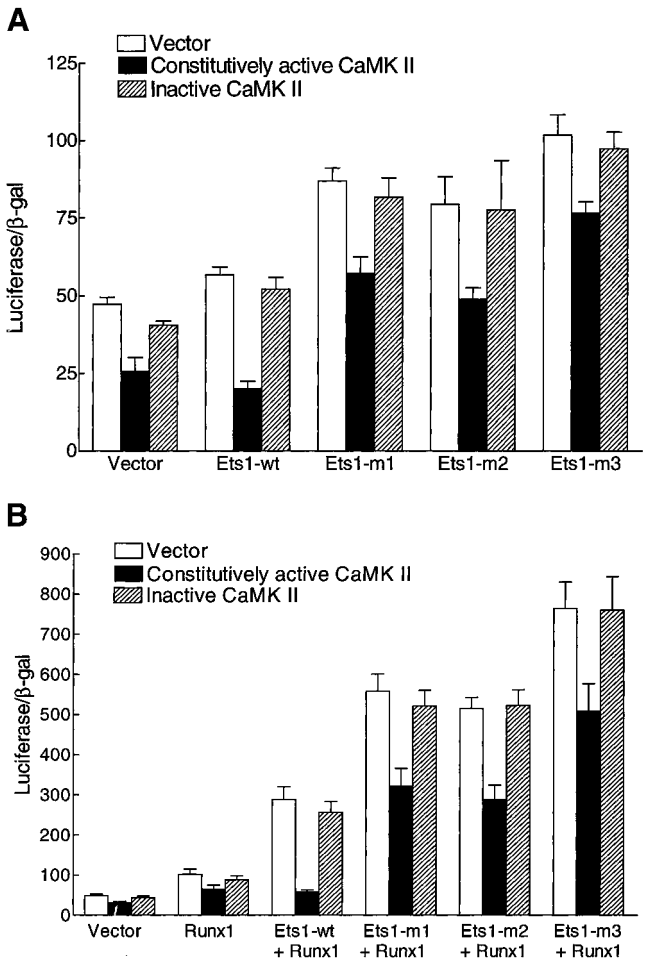

Figure 6. Decreased inhibition of the GM-CSF promoter/enhancer by constitutively active CaMK II on mutation of Ets1. (A) Cotransfection of constitutively active CaMK II T286D, inactive CaMK II T286A, or vector control with Ets1 wild type, mutant Ets1, or vector control in DG75 cells. (B) Same as A except that Runx1 was also cotransfected. Bars represent average ratio of luciferase/β-galactosidase activity in arbitrary units from three independent transfections ± SD, using β-galactosidase expression from an hCMV-β-gal plasmid for normalization.

stitutively active CaMK II when Runx1 was coexpressed. As shown in Figure 6B, constitutively active CaMK II could efficiently inhibit the synergism of Runx1 with wild-type Ets1. This inhibitory effect of constitutively active CaMK II was dramatically decreased by the Ets1 mutations even in the presence of Runx1. Compared with the wild-type, Ets1-m1, Ets1-m2, and Ets1-m3 gave 5.5-, 4.9-, and 8.7-fold higher transcriptional activity, respectively, in the presence of Runx1 and constitutively active CaMK II. These results show that inhibition of Ets1 by CaMK II also occurs when Ets1 cooperates with Runx1, which has the potential to relieve autoinhibition of Ets1.

We also analyzed the effect of overexpression of constitutively active calcineurin. As expected, calcineurin increased transcription of the GM-CSF reporter. None of the Ets1-m1,

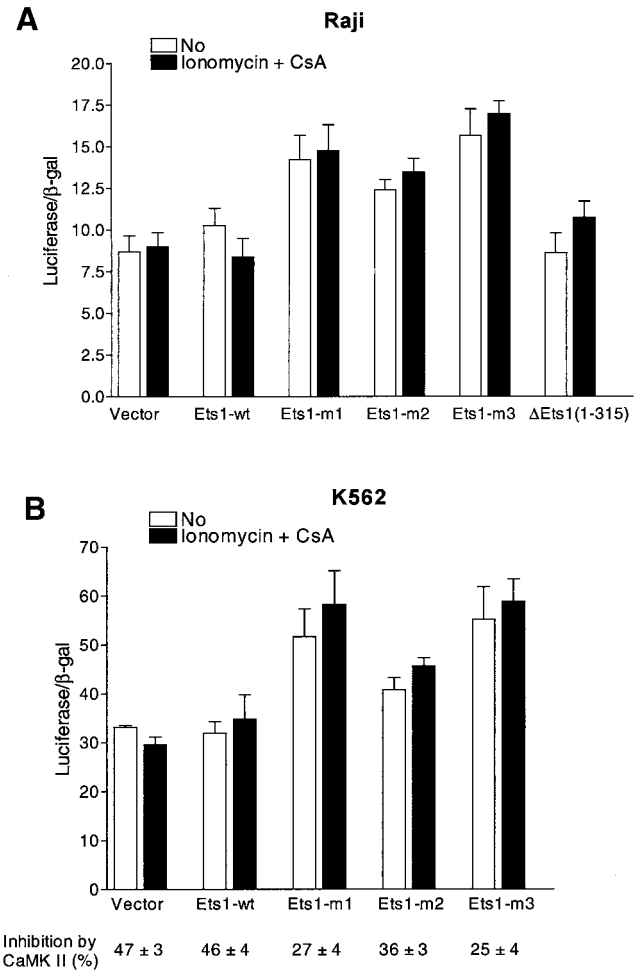

Figure 7. Effects of Ets1 wild-type and mutants on the GM-CSF promoter/enhancer in (A) Raji cells and (B) K562 cells in the absence and presence of ionomycin plus CsA. Bars represent average ratio of luciferase/β-galactosidase activity in arbitrary units from three independent transfections ± SD, using β-galactosidase expression from an hCMV-β-gal plasmid for normalization. The results from cotransfections of Ets1 wild type, mutant Ets1, or vector control with constitutively active CaMK II T286D compared with vector control are shown below the bars in B. These numbers are the average inhibitions in per cent from three independent transfections ± SD.

Ets1-m2, or Ets1-m3 mutations had any effect on the activation by calcineurin (data not shown). Thus, none of the analyzed CaMK II sites in Ets1 contribute significantly to the positive effect of calcineurin on the GM-CSF promoter/enhancer.

To analyze whether Ca^{2+} inhibition of Ets1 regulates GM-CSF transcription only in DG75 cells or in a broad range of cells, we analyzed the effects of the Ets1 mutations in the absence and presence of ionomycin plus CsA in other cell lines as well. The Ets1 mutations increased transcription of the GM-CSF reporter by Ets1 in all analyzed cell lines. The results with the B-lymphocyte cell line Raji and the myeloid cell line K562 are shown in Figure 7. In the Raji cell line, the mutations of Ets1 increased the activation of the GM-CSF reporter much more when the cells were treated with ionomycin plus CsA than without the treatment, showing that inhibition of wild-type Ets1 was Ca^{2+} dependent in this cell line as well (Figure 7A). However, in K562 cells, the increase in GM-CSF reporter activation was approximately equal in the presence and absence of ionomycin plus CsA treatment. The most likely explanation for the difference between the cell lines was a limiting CaMK II activity in K562 cells, which would lead to a lack of inhibitory effect of ionomycin plus CsA treatment. This was indeed the case, because expression of constitutively active CaMK II resulted in a strong inhibition of the GM-CSF reporter in Ets1-wt–transfected K562 cells but a much smaller inhibition of the reporter in cells transfected with mutant Ets1 (Figure 7B, bottom). The decrease in the inhibition by constitutively active CaMK II was from 46 ± 4% in Ets1-wt transfected K562 to only 25 ± 4% inhibition in the Ets1-m3–transfected cells. Transfection of Jurkat cells with Ets1-wt gave a low level of GM-CSF reporter transcription that also was more inhibited by cotransfection of CaMK II than the higher level of transcription in Ets1-m3 transfected Jurkat cells (data not shown).

To analyze whether the Ets domain containing part of Ets1 was needed for the decrease in GM-CSF reporter activation by wild-type Ets1 on ionomycin plus CsA treatment, we constructed a C-terminal deletion derivative of the Ets1 expression plasmid, ΔEts1(1–315). This deletion derivative was included in the analysis in Raji cells, in which, as in DG75 cells, ionomycin plus CsA treatment had a negative effect on GM-CSF activation on expression of Ets1-wt. The deletion was found to block the negative effect of ionomycin plus CsA, and this treatment even led to a small increase in transcription (Figure 7A). Furthermore, this deletion of Ets1 resulted in loss of most of the inhibitory effect of ionomycin plus CsA treatment or of CaMK II overexpression in DG75 cells (data not shown). This shows that the Ets domain or a sequence C-terminal to it is needed for the inhibition of GM-CSF transcription by Ca^{2+} activation of CaMK II inhibition of Ets1.

CaMK II Inhibits Ets1 Binding to the GM-CSF Promoter In Vivo

The GM-CSF promoter contains several Ets1 binding sites (Figure 8A), and transactivation of the GM-CSF promoter by Ets1 requires interaction of Ets1 with at least one intact Ets1 binding site, GM5 (Thomas *et al.*, 1995; Thomas *et al.*, 1997; McKinlay *et al.*, 1998). To examine whether Ca^{2+} signaling and phosphorylation by CaMK II affects Ets1 binding to the promoter in vivo, ChIP PCR analysis was performed. A pair of primers flanking the strongest Ets1 binding sites in the GM-CSF promoter was used (Figure 8A). The results in Figure 8B show that treatment with either ionomycin plus CsA or overexpression of constitutively active CaMK II led to a strong reduction of in vivo binding of transfected Ets1 to the DNA segment containing the strongest Ets1 binding sites in the GM-CSF promoter of the reporter plasmid. No significant effect by either ionomycin plus CsA or coexpression of CaMK II was observed when the mutant Ets1-m3 was expressed, showing that the CaMK II phosphorylation sites were important for the reduction in the binding of Ets1 to the promoter in vivo.

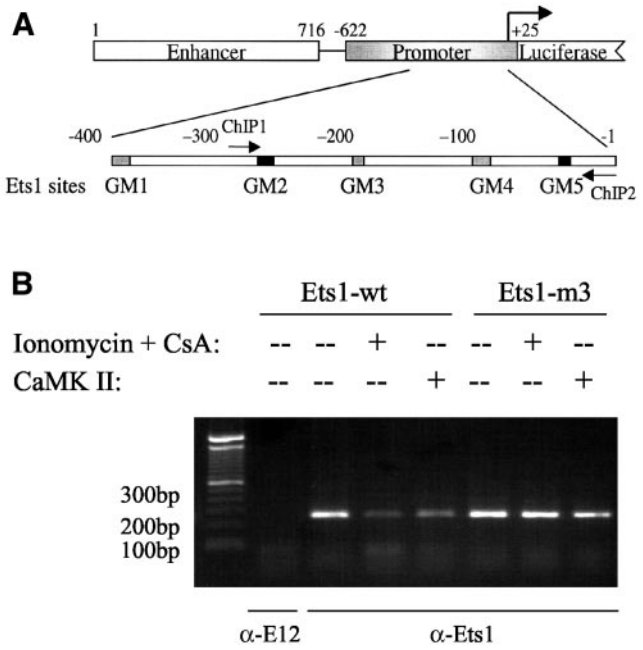

Figure 8. Binding of wild-type and mutant Ets1 to the GM-CSF promoter in vivo: decreased binding of wild-type Ets1 but not the Ets1-m3 mutant on ionomycin plus CsA treatment or expression of constitutively active CaMK II. (A) Schematic drawing of the GM-CSF reporter plasmid. The 716-bp human GM-CSF enhancer and the 647-bp human GM-CSF promoter segments (Cockerill *et al.*, 1993; Jenkins *et al.*, 1995; Shannon *et al.*, 1997) are indicated. The positions of the primers used in ChIP, ChIP1 and ChIP2, relative to the weak (gray) and strong (black) Ets1 binding sites (Thomas *et al.*, 1995) are indicated. (B) The amounts of the PCR product after ChIP with wild-type and mutant Ets1. Where indicated, either the cells were treated with ionomycin plus CsA, or constitutively active CaMK II was expressed (MATERIALS AND METHODS).

DISCUSSION

A broad range of cells, including T lymphocytes and mantle zone B lymphocytes, produce GM-CSF in response to different immune-activating and inflammatory stimuli (Gasson, 1991; Pistoia and Corcione, 1995). One major signal in T cells responding to TCR activation is an increase in intracellular Ca^{2+}. Activation of GM-CSF expression by Ca^{2+} occurs through the phosphatase calcineurin (Shannon *et al.*, 1997). Other immune and inflammatory stimuli, such as BCR activation, also bring about signaling by the second messenger Ca^{2+} (Healy *et al.*, 1997; Benschop *et al.*, 2001). BCR activation of B lymphocytes and TCR activation of T lymphocytes lead to Ca^{2+}-dependent phosphorylation of Ets1 (Pognonec *et al.*, 1990; Fisher *et al.*, 1991; Rabault and Ghysdael, 1994). In the present study, we have shown that Ca^{2+} signaling not only positively regulates transcription from the GM-CSF promoter/enhancer through calcineurin activation of the transcription factors NF-AT, NF-κB, and AP-1 but also negatively regulates it through CaMK II phosphorylation of serines in the auto-inhibitory domain for DNA binding of the transcription factor Ets1 (Figure 9).

It is interesting that the Ca^{2+} second messenger can elicit both positive and negative effects on transcription of the same gene. Even though Ca^{2+} is a ubiquitous second messenger, Ca^{2+} signals lead to GM-CSF expression in only a small fraction of all cell types, including T cells and certain B cells. Furthermore, a positive Ca^{2+} signaling pathway dependent on calcineurin combined with a negative pathway dependent on CaMK II (Figure 9) would lead to a Ca^{2+} activation of GM-CSF transcription only when the activity or quantity of components of the former pathway dominate over those of the latter pathway. Moreover, Ca^{2+} signaling after BCR or TCR activation displays both amplitude and frequency modulation. Foreign antigen triggers a large biphasic Ca^{2+} response in naive B cells, whereas tolerant B cells display an increased basal Ca^{2+} level and the self-antigen stimulates low Ca^{2+} oscillations (Healy *et al.*, 1997). Similarly, T cells induced to anergy display an elevated basal Ca^{2+} concentration, and comparatively low-amplitude Ca^{2+} responses are found when T-cell anergy is induced by altered peptide ligands (Gajewski *et al.*, 1990, 1994a,b; Sloan-Lancaster *et al.*, 1996). T cells stimulated through the TCR display Ca^{2+} oscillations with a period of ~100 s (Gajewski *et al.*, 1990, 1994a,b; Sloan-Lancaster *et al.*, 1996). It is also notable in this context that immature B cells have a higher-amplitude Ca^{2+} response to antigen than mature B cells and that α-hemolysin of uropathogenic *E. coli* induces Ca^{2+} oscillations with a period of ~700 s in renal epithelial cells (Uhlen *et al.*, 2000; Benschop *et al.*, 2001). Therefore, because differential Ca^{2+} signaling plays a key role in the distinct responses to self and nonself, it is not surprising that transcription of a cytokine can be both positively and negatively regulated by Ca^{2+}. The importance of plasticity in the Ca^{2+} signaling system is in line with the participation in GM-CSF regulation of at least three calcineurin-activated transcription factors, NF-AT, AP-1, and NF-κB (Shannon *et al.*, 1997; Shang *et al.*, 1999), that each display a different pattern of amplitude and frequency modulation in response to Ca^{2+} signaling (Dolmetsch *et al.*, 1997, 1998). Moreover, the frequency of intracellular Ca^{2+} oscillations can be decoded through CaMK II, because its activity is highly sensitive to the temporal pattern of Ca^{2+} oscillations, and CaMK II is known to be a key mediator of many Ca^{2+} effects (Dupont and Goldbeter, 1998; Lukas *et al.*, 1998).

Wild-type Ets1 negatively affected GM-CSF transcription on Ca^{2+} stimulation of DG75 and Raji cells in the presence of a calcineurin inhibitor, whereas Ets1 with mutated CaMK II target serines resulted in increased transactivation of GM-CSF. Likewise, wild-type Ets1 but not mutant Ets1 had a negative effect in DG75 cells when constitutively active CaMK II was expressed. The negative effect of wild-type Ets1 suggests that it functions as a dominant inhibitory protein that decreases transactivation by a functionally related protein(s) (Figure 9). Such a related protein could be the p42 splice form of Ets1 (Figure 9) that lacks the autoinhibitory exon VII domain (Koizumi *et al.*, 1990; Wasylyk *et al.*, 1992; Fisher *et al.*, 1994). There is also a large family of Ets1-related Ets transcription factors that can function as either transcriptional activators or repressors (Mimeault, 2000; Yordy and Muise-Helmericks, 2000; Lelievre *et al.*, 2001). Significantly, the DG75 cell line contains large amounts of proteins binding to DNA sites for Ets family members in EMSA (Nilsson *et al.*, 1995). Therefore, an alternative possibility is that another Ets family member, less inhibited by CaMK II, contributes to GM-CSF transcription,

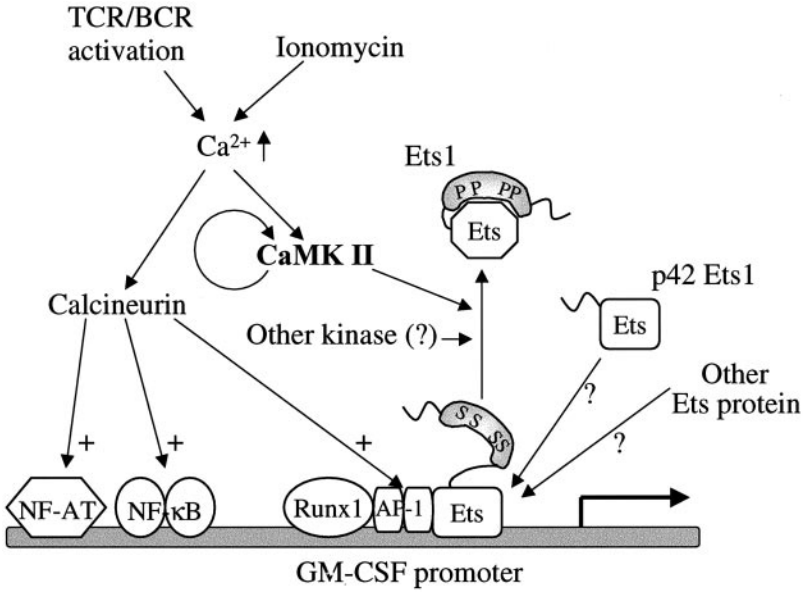

Figure 9. Schematic illustrating the discussed models for regulation of GM-CSF transcription. The binding of NF-AT, NF-κB, Ets1, AP-1, and Runx1 transcription factors to the promoter is shown. For simplicity, only one NF-AT site and one Ets1 site are drawn, although the GM-CSF promoter/enhancer contains multiple binding sites for these factors (Thomas *et al.*, 1995; Shannon *et al.*, 1997; Thomas *et al.*, 1997; McKinlay *et al.*, 1998; Shang *et al.*, 1999). The autoinhibitory domain of Ets1 (gray) and the Ets domain are also shown. Nonphosphorylated serines at positions 251, 257, 282, and 285 in the autoinhibitory domain are indicated with S and phosphorylation of these serines with P. Ca^{2+} signaling, which can be induced by TCR or BCR activation or by ionomycin treatment, positively regulates transcription from the GM-CSF promoter/enhancer through calcineurin activation of the transcription factors NF-AT, NF-κB, and AP-1 (Shannon *et al.*, 1997). In the present study, we show that Ca^{2+} signaling can also negatively regulate the GM-CSF promoter/enhancer through CaMK II phosphorylation of serines in the autoinhibitory domain for DNA binding of Ets1. Serine phosphorylation in the autoinhibitory domain stabilizes the conformation that inhibits the DNA binding of the Ets domain (Cowley and Graves, 2000). The negative effect of wild-type Ets1 suggests that it functions as a dominant inhibitory protein that decreases transactivation by a functionally related protein(s), such as the p42 splice form of Ets1, that lacks the autoinhibitory domain or another Ets family member(s) that is less inhibited by CaMK II. The finding that the mutations also increased the Ets1 activity in the absence of Ca^{2+}/CaMK II–activating treatment may, as illustrated, indicate that another kinase can mediate a partial phosphorylation in the absence of Ca^{2+} signaling. The circular arrow indicates a possible dynamic balance between Ca^{2+}-dependent CaMK II and autophosphorylation that makes CaMK II independent of Ca^{2+} (Lukas *et al.*, 1998). The intermediate nucleotides between the Runx1 and Ets1 sites in the GM-CSF promoter constitute a binding site for AP-1. The results suggest that when Ets1 functions in cooperation with nearby AP-1, at least a large part of the autoinhibition through phosphorylation of the autoinhibitory domain remains and is not relieved by interaction between Ets1 and Runx1.

at least when Ets1 is Ca^{2+} inhibited (Figure 9). Such a contribution could also explain why phosphorylation of the four inhibitory CaMK II sites decreases DNA binding of Ets1 50-fold in vitro (Cowley and Graves, 2000), whereas expression of this mutant gave a 6- to 9-fold higher increase in transcription than the wild type on Ca^{2+} stimulation in the presence of CsA or expression of constitutively active CaMK II (Figures 4B and 6A). We also found that the Ca^{2+}-dependent phosphorylation of Ets1 reduced the DNA binding of Ets1 to the GM-CSF promoter in vivo to a level that resembles the decrease in GM-CSF transcription in vivo (compare Figures 4B, 6A, and 8).

Transfection analysis of Ets1 with deletion of the C-terminal part, containing the DNA binding Ets domain, showed that the Ets domain or a sequence C-terminal to it is needed for the inhibition by Ca^{2+} activation in the presence of calcineurin inhibitor (Figure 7). However, the Ets domain is not only interacting with DNA but is also one of the domains participating in the interaction enabling the synergy with Runx1 (Kim *et al.*, 1999), which is important for GM-CSF expression (Figures 5 and 6B). Perhaps the Ets domain also participates in another relevant protein–protein interaction(s), because Ets1 binds DNA cooperatively with several binding partners, including NF-κB and the AP-1 proteins Jun/Fos (references in Cowley and Graves, 2000). Thus, either the DNA binding of Ets1 or a protein interaction site in the Ets domain or on its C-terminal side is needed for the dominant interference.

We conclude that interaction of another protein(s) with Ets1 and/or with the promoter in vivo decreases the negative effect of the Ets1 phosphorylations, or alternatively, the Ca^{2+}/CaMK II–activating treatments in vivo caused a quantitative but not a qualitative change in the phosphorylation of these sites. The latter alternative is supported by the finding that the mutations also increased the Ets1 activity, although to a lower extent, in the absence of Ca^{2+}/CaMK II–activating treatment. This may indicate that another kinase can mediate a partial phosphorylation in the absence of Ca^{2+} signaling. Yet another alternative could be that there is a dynamic balance between Ca^{2+}-dependent CaMK II and autophosphorylation that makes CaMK II independent of Ca^{2+} (Lukas *et al.*, 1998). The discussed models for regulation of the effect of Ets1 on GM-CSF transcription are schematically illustrated in Figure 9.

Binding of the Runx1 transcription factor has been reported to facilitate Ets1 DNA binding at the Tβ3 and Tβ4 elements in the TCRβ enhancer and at the transcription control region of murine leukemia virus by counteracting autoinhibition of Ets1 (Kim *et al.*, 1999; Goetz *et al.*, 2000). Nevertheless, mutation of CaMK II target serines in Ets1 relieved inhibition of the cooperative transactivation of GM-CSF by Ets1 and Runx1 (Figures 5 and 6B). Presumably, the reason for the apparent difference between our results and those of the previous studies is not the slightly higher number of nucleotides between the Runx1 and Ets1 binding sites at the GM-CSF promoter, because the relief of autoinhibition was unaffected even if the distance between the two sites was expanded so that it became greater than that present in the GM-CSF promoter (Goetz *et al.*, 2000). The main difference between the Runx1 and Ets1 sites of the GM-CSF pro-

moter and the other promoters/enhancers is that the intermediate nucleotides in the GM-CSF promoter constitute a binding site for an AP-1 transcription factor (Figure 9), and together, the AP-1 and Ets binding sites constitute the important CLE0 element of the promoter (Shannon *et al.*, 1997; Thomas *et al.*, 1997). Our results suggest that when Ets1 functions in cooperation with nearby AP-1, then at least a large part of the autoinhibition through phosphorylation of the exon VII domain remains and is not relieved by overexpression of Runx1. It is notable in this context that the interaction of Ets1 with Runx1 is to a large extent through the autoinhibitory exon VII domain (Kim *et al.*, 1999), and it is therefore possible that exon VII phosphorylation has an effect on this interaction.

In summary, we have shown that Ca^{2+} has a dual role in positively regulating GM-CSF through calcineurin and negatively through Ets1 phosphorylation by CaMK II. The role of this regulatory mechanism for the plasticity in the amplitude- and frequency-modulated immune and inflammatory Ca^{2+} responses is an important issue for future studies.

ACKNOWLEDGMENTS

This work was supported by a grant from the Swedish Cancer Society.

REFERENCES

Benschop, R.J., Brandl, E., Chan, A.C., and Cambier, J.C. (2001). Unique signaling properties of B cell antigen receptor in mature and immature B cells: implications for tolerance and activation. J Immunol. *167*, 4172–4179.

Bories, J.C., Willerford, D.M., Grevin, D., Davidson, L., Camus, A., Martin, P., Stehelin, D., and Alt, F.W. (1995). Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. Nature *377*, 635–638.

Cockerill, P.N., Shannon, M.F., Bert, A.G., Ryan, G.R., and Vadas, M.A. (1993). The granulocyte-macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible cyclosporin A-sensitive enhancer. Proc. Natl. Acad. Sci. USA *90*, 2466–2470.

Cowley, D.O., and Graves, B.J. (2000). Phosphorylation represses Ets-1 DNA binding by reinforcing autoinhibition. Genes Dev. *14*, 366–376.

Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C., and Healy, J.I. (1997). Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. Nature *386*, 855–858.

Dolmetsch, R.E., Xu, K., and Lewis, R.S. (1998). Calcium oscillations increase the efficiency and specificity of gene expression. Nature *392*, 933–936.

Dupont, G., and Goldbeter, A. (1998). CaM kinase II as frequency decoder of Ca^{2+} oscillations. Bioessays *20*, 607–610.

Estrov, Z., Talpaz, M., Ku, S., Harris, D., LaPushin, R., Koller, C.A., Hirsh-Ginsberg, C., Huh, Y., Yee, G., Kurzrock, R. (1996a). Molecular and biologic characterization of a newly established Philadelphia-positive acute lymphoblastic leukemia cell line (Z-33) with an autocrine response to GM-CSF. Leukemia *10*, 1534–1543.

Estrov, Z., Talpaz, M., Ku, S., Harris, D., Van, Q., Beran, M., Hirsch-Ginsberg, C., Huh, Y., Yee, G., Kurzrock, R. (1998). Z-138: a new mature B-cell acute lymphoblastic leukemia cell line from a patient with transformed chronic lymphocytic leukemia. Leuk. Res. *22*, 341–353.

Estrov, Z., Talpaz, M., Zipf, T.F., Kantarjian, H.M., Ku, S., Ouspenskaia, M.V., Hirsch-Ginsberg, C., Huh, Y., Yee, G., Kurzrock, R. (1996b). Role of granulocyte-macrophage colony-stimulating factor in Philadelphia (Ph1)-positive acute lymphoblastic leukemia: studies on two newly established Ph1-positive acute lymphoblastic leukemia cell lines (Z-119 and Z-181). J. Cell Physiol. *166*, 618–630.

Feske, S., Draeger, R., Peter, H.H., Eichmann, K., and Rao, A. (2000). The duration of nuclear residence of NFAT determines the pattern of cytokine expression in human SCID T cells. J. Immunol. *165*, 297–305.

Fisher, C.L., Ghysdael, J., and Cambier, J.C. (1991). Ligation of membrane Ig leads to calcium-mediated phosphorylation of the proto-oncogene product, Ets-1. J. Immunol. *146*, 1743–1749.

Fisher, R.J., Fivash, M., Casas-Finet, J., Erickson, J.W., Kondoh, A., Bladen, S.V., Fisher, C., Watson, D.K., and Papas, T. (1994). Real-time DNA binding measurements of the ETS1 recombinant oncoproteins reveal significant kinetic differences between the p42 and p51 isoforms. Protein Sci. *3*, 257–266.

Gajewski, T.F., Lancki, D.W., Stack, R., and Fitch, F.W. (1994a). "Anergy" of TH0 helper T lymphocytes induces downregulation of TH1 characteristics and a transition to a TH2-like phenotype. J. Exp. Med. *179*, 481–491.

Gajewski, T.F., Qian, D., Fields, P., and Fitch, F.W. (1994b). Anergic T-lymphocyte clones have altered inositol phosphate, calcium, and tyrosine kinase signaling pathways. Proc. Natl. Acad. Sci. USA *91*, 38–42.

Gajewski, T.F., Schell, S.R., and Fitch, F.W. (1990). Evidence implicating utilization of different T cell receptor-associated signaling pathways by TH1 and TH2 clones. J. Immunol. *144*, 4110–4120.

Gasson, J.C. (1991). Molecular physiology of granulocyte-macrophage colony-stimulating factor. Blood *77*, 1131–1145.

Goetz, T.L., Gu, T.L., Speck, N.A., and Graves, B.J. (2000). Autoinhibition of Ets-1 is counteracted by DNA binding cooperativity with core-binding factor alpha2. Mol. Cell. Biol. *20*, 81–90.

Healy, J.I., Dolmetsch, R.E., Timmerman, L.A., Cyster, J.G., Thomas, M.L., Crabtree, G.R., Lewis, R.S., and Goodnow, C.C. (1997). Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. Immunity *6*, 419–428.

Hughes, K., Antonsson, Å., and Grundström, T. (1998). Calmodulin dependence of NFkappaB activation. FEBS Lett. *441*, 132–136.

Jenkins, F., Cockerill, P.N., Bohmann, D., and Shannon, M.F. (1995). Multiple signals are required for function of the human granulocyte-macrophage colony-stimulating factor gene promoter in T cells. J. Immunol. *155*, 1240–1251.

Kim, W.Y., Sieweke, M., Ogawa, E., Wee, H.J., Englmeier, U., Graf, T., and Ito, Y. (1999). Mutual activation of Ets-1 and AML1 DNA binding by direct interaction of their autoinhibitory domains. EMBO J. *18*, 1609–1620.

Koizumi, S., Fisher, R.J., Fujiwara, S., Jorcyk, C., Bhat, N.K., Seth, A., and Papas, T.S. (1990). Isoforms of the human ets-1 protein: generation by alternative splicing and differential phosphorylation. Oncogene *5*, 675–681.

Lars, N., and Paschalis, S. (1993). The human I alpha 1 and I alpha 2 germline promoter elements: proximal positive and distal negative elements may regulate the tissue specific expression of C alpha 1 and C alpha 2 germline transcripts. Int. Immunol. *5*, 271–282.

Lelievre, E., Lionneton, F., Soncin, F., and Vandenbunder, B. (2001). The Ets family contains transcriptional activators and repressors involved in angiogenesis. Int. J. Biochem. Cell Biol. *33*, 391–407.

Liles, W.C. (2001). Immunomodulatory approaches to augment phagocyte-mediated host defense for treatment of infectious diseases. Semin. Respir. Infect. *16*, 11–17.

Lukas, T.J., Mirzoeva, S., and Watterson, D.M. (1998). Calmodulin-regulated protein kinases. In: Calmodulin and Signal Transduction, eds. L. Van Eldik and D.M. Watterson, New York: Academic Press, 65–168.

Lutterbach, B., and Hiebert, S.W. (2000). Role of the transcription factor AML-1 in acute leukemia and hematopoietic differentiation. Gene *245*, 223–235.

McKinlay, L.H., Tymms, M.J., Thomas, R.S., Seth, A., Hasthorpe, S., Hertzog, P.J., and Kola, I. (1998). The role of Ets-1 in mast cell granulocyte-macrophage colony-stimulating factor expression and activation. J. Immunol. *161*, 4098–4105.

Mimeault, M. (2000). Structure-function studies of ETS transcription factors. Crit. Rev. Oncog. *11*, 227–253.

Muthusamy, N., Barton, K., and Leiden, J.M. (1995). Defective activation and survival of T cells lacking the Ets-1 transcription factor. Nature *377*, 639–642.

Nghiem, P., Saati, S.M., Martens, C.L., Gardner, P., and Schulman, H. (1993). Cloning and analysis of two new isoforms of multifunctional Ca^{2+}/calmodulin-dependent protein kinase: expression in multiple human tissues. J. Biol. Chem. *268*, 5471–5479.

Nilsson, L., Grant, P., Larsson, I., Pettersson, S., and Paschalis, S. (1995). The human $I_{\alpha}1$ region contains a TGF-β1 responsive enhancer and a putative recombination hotspot. Int. Immunol. *7*, 1191–1204.

Orlando, V., Strutt, H., and Paro, R. (1997). Analysis of chromatin structure by in vivo formaldehyde cross-linking. Methods *11*, 205–214.

Pistoia, V., and Corcione, A. (1995). Relationships between B cell cytokine production in secondary lymphoid follicles and apoptosis of germinal center B lymphocytes. Stem Cells *13*, 487–500.

Pognonec, P., Boulukos, K.E., Bosselut, R., Boyer, C., Schmitt-Verhulst, A.M., and Ghysdael, J. (1990). Identification of a Ets1 variant protein unaffected in its chromatin and in vitro DNA binding capacities by T cell antigen receptor triggering and intracellular calcium rises. Oncogene *5*, 603–610.

Rabault, B., and Ghysdael, J. (1994). Calcium-induced phosphorylation of ETS1 inhibits its specific DNA binding activity. J. Biol. Chem. *269*, 28143–28151.

Shang, C., Attema, J., Cakouros, D., Cockerill, P.N., and Shannon, M.F. (1999). Nuclear factor of activated T cells contributes to the function of the CD28 response region of the granulocyte macrophage-colony stimulating factor promoter. Int. Immunol. *11*, 1945–1956.

Shannon, M.F., Coles, L.S., Vadas, M.A., and Cockerill, P.N. (1997). Signals for activation of the GM-CSF promoter and enhancer in T cells. Crit. Rev. Immunol. *17*, 301–323.

Sloan-Lancaster, J., Steinberg, T.H., and Allen, P.M. (1996). Selective activation of the calcium signaling pathway by altered peptide ligands. J. Exp. Med. *184*, 1525–1530.

Thomas, R.S., Tymms, M.J., McKinlay, L.H., Shannon, M.F., Seth, A., and Kola, I. (1997). ETS1, NFkappaB and AP1 synergistically transactivate the human GM-CSF promoter. Oncogene *14*, 2845–2855.

Thomas, R.S., Tymms, M.J., Seth, A., Shannon, M.F., and Kola, I. (1995). ETS1 transactivates the human GM-CSF promoter in Jurkat T cells stimulated with PMA and ionomycin. Oncogene *11*, 2135–2143.

Tracey, W.D., and Speck, N.A. (2000). Potential roles for RUNX1 and its orthologs in determining hematopoietic cell fate. Semin. Cell Dev. Biol. *11*, 337–342.

Uhlen, P., Laestadius, A., Jahnukainen, T., Soderblom, T., Backhed, F., Celsi, G., Brismar, H., Normark, S., Aperia, A., Richter-Dahlfors, A. (2000). Alpha-hemolysin of uropathogenic *E. coli* induces Ca^{2+} oscillations in renal epithelial cells. Nature *405*, 694–697.

Wasylyk, C., Kerckaert, J.P., and Wasylyk, B. (1992). A novel modulator domain of Ets transcription factors. Genes Dev. *6*, 965–974.

Xie, X.Q., Pardali, E., Holm, M., Sideras, P., and Grundström, T. (1999). AML and Ets proteins regulate the I alpha1 germ-line promoter. Eur. J. Immunol. *29*, 488–498.

Yordy, J.S., and Muise-Helmericks, R.C. (2000). Signal transduction and the Ets family of transcription factors. Oncogene *19*, 6503–6513.