Rapid and transient expression of Ets2 in mature macrophages following stimulation with cMGF, LPS, and PKC activators

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We reported previously that Ets2 is expressed in normal and transformed macrophages. We show here that the expression of both *c-ets-2* mRNA and proteins is induced rapidly and transiently in chicken nondividing bone marrow-derived macrophages but not in E26-transformed myeloblasts in response to chicken myelomonocytic growth factor (cMGF), an avian hematopoietic growth factor required for survival, proliferation, and colony formation of avian myeloid cells. *c-ets-2* expression is also rapidly induced in chicken bone marrow-derived macrophages, human monocytes, and mouse peritoneal macrophages in response to LPS and/or PKC activators. The rapid induction of Ets2 after treatment of chicken bone marrow-derived macrophages by cMGF is blunted after down-regulation or inactivation of PKC, suggesting a role of PKC in the cMGF-induced signal transduction pathway. Because Ets2 is localized in the nucleus of macrophages and binds to DNA in vitro, the kinetics of its expression suggest a role for Ets2 in the transduction within the nucleus of specific signals received at the cell membrane and involved in securing the survival and/or the development of functional competence of these cells.

[Key Words: *c-ets-2*; proto-oncogene; PKC; cMGF; macrophages; early response genes]

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E26 is a replication-defective retrovirus that causes erythroblastosis and myeloblastosis in chickens and transforms erythroblasts and myeloblasts in culture (Graf et al. 1979; Moscovici et al. 1981, 1983; Radke et al. 1982). Together with a partial *gag* gene, the genome of E26 contains the oncogenes *v-myb*^E (Roussel et al. 1979) and *v-ets* (Leprince et al. 1983; Nunn et al. 1983), which are both implicated in the oncogenic potential of E26 (Beug et al. 1984; Golay et al. 1988; Nunn and Hunter 1989). The *c-ets* gene family includes genes with sequence identity to the *v-ets* oncogene and consists of *c-ets-1* (Chen 1985; Watson et al. 1985; Ghysdael et al. 1986a), the progenitor to *v-ets* (Gegonne et al. 1987; Chen 1988; Duterque-Coquillaud et al. 1988; Leprince et al. 1988), *c-ets-2* (Watson et al. 1985; Ghysdael et al. 1986b; Boulukos et al. 1988), *erg* (Rao et al. 1987; Reddy et al. 1987), and *elk* (Rao et al. 1989). The chicken 54-kD *c-ets-1* (Ets1) and 58- to 64-kD *c-ets-2* (Ets2) gene

products are nuclear phosphoproteins (Boulukos et al. 1988; Pognonec et al. 1988, 1989) that share two domains of identity of 96 and 175 amino acids in their amino- and carboxy-terminal domains, respectively (Boulukos et al. 1988).

Ets1 is a nuclear chromatin-associated protein (Pognonec et al. 1989) highly expressed in lymphoid cells and tissues (Chen 1985; Ghysdael et al. 1986a), which displays DNA-binding capacity in vitro (Pognonec et al. 1989). Stimulation of thymocytes with mitogenic doses of concanavalin A results in the rapid Ca²⁺-dependent phosphorylation of Ets1 (Pognonec et al. 1988). These Ca²⁺-dependent phosphorylation events result in both a loss of affinity of Ets1 for chromatin and its ability to bind to DNA in vitro (Pognonec et al. 1989), suggesting a role for Ets1 as a nuclear mediator of the Ca²⁺ signal transduction pathway in lymphoid cells.

The *c-ets-2* gene is expressed in a wide variety of tissues (Bhat et al. 1987), and its expression has been proposed to be intrinsically linked to cell proliferation in mouse regenerating liver and fibroblasts (Bhat et al. 1987). We reported previously that Ets2 expression is prominent in avian macrophages and that synthesis of these proteins is induced upon 12-O-tetradecanoyl-

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phorbol 13-acetate (TPA)-induced differentiation of AMV-transformed monoblasts into macrophage-like cells (Ghysdael et al. 1986b). Here we show that *c-ets-2* is rapidly and transiently induced in chicken nondividing bone marrow-derived macrophages (BMM) in response to an avian hematopoietic growth factor, chicken myelomonocytic growth factor (cMGF), required for survival, proliferation, and colony formation of avian myeloid cells, as well as to bacterial lipopolysaccharide (LPS) and protein kinase C (PKC) activators. Ets2 is localized in the nucleus of macrophages and, like Ets1, displays DNA binding activity in vitro. These properties show that in macrophages, transient *c-ets-2* expression is not necessarily linked to cellular proliferation but could be involved in cellular survival and/or the development of functional competence.

Results

Induction of *c-ets-2* gene expression in chicken macrophages

We reported previously that Ets2 is expressed in chicken BMM and that its synthesis is specifically induced upon treatment of AMV-transformed monoblasts with the phorbol ester TPA (Ghysdael et al. 1986b). Because signal transduction by TPA involves direct activation of PKC in cells, we hypothesized that the induction of Ets2 by TPA in mature cells of the myelomonocytic lineage could reflect an event bypassing a ligand–receptor interaction occurring at the cell surface. The only well-characterized avian hematopoietic stimulating factor in myeloid cells is cMGF, a 201-amino-acid glycoprotein required for growth and survival of these cells and for colony formation of myeloid precursor cells (Leutz et al. 1984, 1989). cMGF is distinct from known mammalian colony-stimulating factors (CSFs), although a mammalian homolog of cMGF probably exists (Leutz et al. 1989). Therefore, we decided to investigate whether cMGF was able to induce *c-ets-2* gene expression in cells of the myeloid lineage. Growth in tissue culture of chicken bone marrow cells in the continuous presence of cMGF results in the proliferation of nonadherent myeloid progenitor cells and in their terminal differentiation into adherent macrophage-like cells (BMM). These adherent BMM form a nongrowing cell population as evidenced by [³H]thymidine incorporation (data not shown), which requires the continuous presence of cMGF for survival. To analyze whether cMGF is a signal sufficient to induce *c-ets-2* gene expression in BMM, cultures were deprived of cMGF for 48 hr and then either left untreated or restimulated with cMGF. After 1 hr, cultures were pulse-labeled with L-[³⁵S]methionine for 20 min and analyzed for Ets2 protein content by immunoprecipitation analysis using an Ets2-specific peptide antiserum (Boulukos et al. 1988) and SDS-PAGE. Whereas stimulation of cMGF-deprived BMM cells with cMGF did not affect total protein synthesis (data not shown), it increased ~10 times the synthesis of Ets2 (Fig. 1A). The four protein species of 58, 60, 62, and 64 kD are no longer detected using the Ets2-specific anti-

Figure 1. (A) Expression of Ets2 in chicken BMM. Adherent, nondividing chicken BMM were deprived of cMGF for 48 hr and cells either were left untreated or were incubated in the presence of nonpurified cMGF (crude cMGF) or a highly purified preparation of cMGF (pur. cMGF; Leutz et al. 1984) or LPS for 1 hr. Cells were pulse-labeled with 500 μ Ci/ml of L-[³⁵S]methionine for 20 min and lysed, and immunoprecipitations were carried out with 10⁷ radioactive counts of each lysate with an Ets2-specific peptide antiserum or with the Ets2-specific peptide antiserum blocked with an excess of the immunogenic peptide (crude cMGF-bl.) and analyzed by SDS-PAGE. (B) RNA blot hybridization. Total RNA (10 μ g) was isolated from untreated chicken BMM deprived of cMGF for 48 hr or chicken BMM deprived of cMGF for 48 hr and restimulated with either cMGF for 1 or 5.5 hr in the absence or presence of actinomycin D or TPA for 1 hr. The RNAs were resolved by formaldehyde-agarose gel electrophoresis, transferred to nylon membranes, and hybridized to ³²P-labeled *c-ets-2* probe or, after stripping, rehybridized to GAPDH as a control.

serum blocked with an excess of the purified peptide immunogen (Fig. 1A) and therefore correspond to the *c-ets-2* gene products identified previously in chicken cells (Ghysdael et al. 1986b; Boulukos et al. 1988). As described previously, the 60- and 64-kD species represent phosphorylated versions of the 58-kD and 62-kD species, which probably result from initiation of translation at two distinct ATGs (initiation codons) (Boulukos et al. 1988). The source of cMGF used in this experiment is a conditioned medium obtained after LPS-stimulation of

the MC29-transformed macrophage cell line HBC-1 (Leutz et al. 1984). LPS, an agent known to stimulate many facets of macrophage functional activation (for review, see Hamilton and Adams 1987), also induces Ets2 expression in BMM (Fig. 1A). To analyze whether the induction of Ets2 in BMM after treatment with the HBC-1-conditioned medium resulted from a true response to cMGF or a response to LPS, we analyzed the effect of a highly purified preparation of cMGF (Leutz et al. 1984). As shown in Figure 1A, treatment of cMGF-depleted BMM with purified cMGF also results in an induction of Ets2 at levels similar to those observed after treatment with either the HD11-conditioned medium or with LPS.

To test whether the purified preparation of cMGF is indeed free of LPS, mouse peritoneal macrophages were selected, as the effects of LPS and cMGF can be separated in this system. Bacterial cell wall products, including LPS, are known to fully activate the functional response of mouse peritoneal macrophages (Adams and Hamilton 1984; Bonvini et al. 1986). In contrast, cMGF does not have a biological activity in murine cells (Leutz et al. 1989). Among the early changes induced by LPS in mouse peritoneal macrophages is the alteration of several competence genes first described in growth factor-stimulated fibroblasts such as *c-fos*, *c-myc*, JE, and KC (Introna et al. 1986, 1987). Thus, we examined *c-fos* protein expression following a 1-hr treatment of mouse peritoneal macrophages with either LPS or purified cMGF. As expected, a 1-hr treatment of mouse peritoneal macrophages with LPS results in the activation of *c-fos* expression, whereas no activation of *c-fos* is observed after a 1-hr treatment with purified cMGF; this demonstrates that the purified cMGF is indeed devoid of LPS (data not shown). In line with the observation in chicken BMM, Ets2 expression is induced by LPS in mouse peritoneal macrophages (Fig. 2). As early as 30 min after LPS treatment of these cells, the level of the 55-kD mouse Ets2 (mo-p55^{c-ets-2}; Pognonec et al. 1989) is increased as detected with an Ets2-specific peptide serum. Two additional Ets2 products (indicated by arrows in Fig. 2) are detected that represent phosphorylated forms of mo-p55^{c-ets-2}, because these forms are converted into the 55-kD species upon alkaline phosphatase treatment (data not shown).

Kinetics of *c-ets-2* induction in cMGF-treated avian macrophages

We next determined the kinetics of chicken *c-ets-2* protein and RNA expression in BMM in response to cMGF. As seen in Figure 3A, cMGF treatment of BMM leads to a rapid increase in Ets2 synthesis, peaking between 1 and 2 hr after cMGF treatment. Furthermore, this increased expression is transient, because the level of Ets2 returns to levels similar to those observed in untreated cells 4.5 hr after cMGF treatment. In contrast to the induction of Ets2, the low level of the 54-kD Ets1 observed in BMM was not found to be affected by treatment with either cMGF or LPS (Fig. 3B).

Consistent with the results presented at the protein

Figure 2. LPS induction of Ets2 in mouse peritoneal macrophages. Twenty-four hours after isolating mouse peritoneal macrophages, cells either were left untreated (control) or were treated with LPS for 30 min (LPS 30 min) or 1 hr (LPS 1 hr). Cells were then pulse-labeled with L-[³⁵S]methionine for 20 min and lysed, and immunoprecipitations were carried out with 10⁷ radioactive counts of each lysate with an Ets2-specific peptide antiserum or with the Ets2-specific peptide antiserum blocked with an excess of the immunogenic peptide (LPS 1h-bl.). Immunoprecipitates were analyzed by SDS-PAGE. The major mouse Ets2 (mo-p55^{c-ets-2}), as well as the slower migrating phosphorylated forms of Ets2, are indicated by arrows.

level, Northern blot analysis showed that treatment of cMGF-starved BMM with cMGF leads to a rapid increase in the level of the 4.0-kb *c-ets-2* RNA species (Fig. 1B). Most of this increase is probably transcriptional, as *c-ets-2* RNA levels are not affected by cMGF treatment in the presence of actinomycin D. In fact, the level of *c-ets-2* RNA appears even lower than that observed in untreated cells, a result reflecting the short half-life (<1 hr) of *c-ets-2* mRNA in macrophages (Fig. 1B; cf. lanes cMGF 1 hr, actD/cMGF 1 hr, and controls; see below).

The level of *c-ets-2* mRNA is also rapidly increased by 1 hr TPA treatment of cMGF-starved BMM (Fig. 1B). Because TPA is a direct activator of PKC and signal transduction by LPS also appears to involve PKC activation (Hamilton and Adams 1987), we investigated whether the induction of the *c-ets-2* gene expression by cMGF in BMM is dependent on the presence of functional PKC. Sustained TPA treatment of cells is known to lead to a down-regulation of PKC (Berridge and Irvine 1984). Therefore, BMM were pretreated with 50 ng/ml of TPA for 54 hr or left untreated as a control, before restimulation of these cells for 1 hr with either a purified preparation of cMGF or 100 ng/ml of TPA, followed by a 30-min pulse-labeling with L-[³⁵S]methionine. In the control samples, an increase in Ets2 synthesis is observed as the result of cMGF or TPA treatment, as expected (Fig. 3C). In contrast, sustained treatment with TPA drastically reduces the ability of cMGF or TPA to induce Ets2 synthesis. In addition, we performed experiments using a potent PKC inhibitor, 1[5-isoquinoliny]l-sulfonyl]-2-

Figure 3. Kinetics of Ets2 induction in chicken BMM and dependence on the presence of a functional PKC. (A and B) BMM were starved of cMGF for 48 hr and restimulated with cMGF for 1, 1.5, 2.5, 4.5, or 8.5 hr. Cells were then pulse-labeled with 500 μ Ci/ml of L-[35 S]methionine for 20 min and lysed, and immunoprecipitations were carried out with 10^7 radioactive counts of each lysate. (A) Lysates were immunoprecipitated with an Ets2-specific peptide antiserum; (B) supernatants from the immunoprecipitation in A were recovered and reimmunoprecipitated with a standard Ets antiserum immunoreactive with both Ets1 and Ets2; (C) after various pretreatments, as indicated, chicken BMM were pulse-labeled and lysed, and immunoprecipitations were carried out with an Ets2-specific peptide antiserum. (Control) Untreated BMM; (-/TPA and -/cMGF) nonpretreated BMM treated with 100 ng/ml TPA or 10 U/ml cMGF, respectively, for 1 hr; (TPA/cMGF and TPA/TPA), BMM pretreated with 50 ng/ml TPA for 54 hr and treated with 10 U/ml cMGF or 100 ng/ml TPA, respectively, for 1 hr; (TPA/-) BMM treated with 50 ng/ml TPA for 54 hr; (H7/cMGF) BMM pretreated with 20 μ M H7 overnight and treated with 10 U/ml cMGF for 1 hr. Unit definition of cMGF is as described by Leutz et al. (1984).

methylpiperazine (H7) (Kawamoto and Hidaka 1984). As shown in Figure 3C, overnight treatment of BMM with 20 μ M H7 before 1-hr stimulation with cMGF leads to a complete block of Ets2 expression. Similar results were obtained when parallel cultures were pretreated with 100 μ M H7 for 2 hr and treated for 1 hr with cMGF (data not shown). Taken together, these results indicate that cMGF activation of *c-ets-2* gene products in BMM can occur via a PKC-dependent pathway.

Treatment of human monocytes by PKC activators results in the rapid expression of *c-ets-2*

TPA treatment of normal human blood-derived monocytes induces these cells to acquire antigenic and biolog-

ical characteristics of macrophages (Kiyotaki and Bloom 1984; Vassali et al. 1984; Todd et al. 1985; Radzioch et al. 1987; Sariban et al. 1987). As shown in Figure 4A, treatment of human peripheral blood monocytes with 33 nM TPA also results in a rapid increase in the 4.7- and 3.2-kb *c-ets-2* RNA transcripts. *c-ets-2* RNA levels are increased as early as 30 min following TPA treatment; however, this induction is not transient as compared with that observed in chicken BMM because it remains high until 12 hr post-treatment (Fig. 4A). Similar results are observed in cMGF or TPA-treated chicken AMV-

Figure 4. Kinetics of *c-ets-2* RNA induction in human monocytes by PKC activators. (A) RNA was isolated from either untreated normal human blood-derived monocytes or normal blood-derived monocytes treated with 33 nM TPA for 30 min or 3, 6, 12, 24, or 72 hr. (B) Normal human blood-derived monocytes were left untreated or treated with 500 nM OAG for 30 min or 3 or 24 hr, before RNA extraction. (C) RNA was isolated from human monocytes treated with either 5 ng/ml actinomycin D alone, 10 μ g/ml cycloheximide alone, or actinomycin D and cycloheximide together for 1, 3, or 6 hr and analyzed as described previously (Sariban et al. 1987), using the 2.3-kb *Eco*RI human *c-ets-2* probe (a generous gift of A. Gégonne). Ribosomal markers are indicated by arrows.

transformed monoblasts (data not shown). Treatment of human monocytes with another PKC activator, the membrane-permeable diacylglycerol, 1-oleoyl-2-acetyl glycerol (OAG), also results in a rapid induction of *c-ets-2* (Fig. 4B). These results demonstrate that activation of the PKC pathway results in activation of the expression of *c-ets-2* in both human monocytes and chicken macrophages.

The half-life of *c-ets-2* RNA in human monocytes is ~1 hr, as determined by chase treatment in the presence of actinomycin D (Fig. 4C). When performed in the presence of cycloheximide, actinomycin D chase experiments show that the *c-ets-2* RNA half-life is increased (Fig. 4C), suggesting a requirement for continuous protein synthesis in the regulation of the short half-life of *c-ets-2* RNA.

c-ets-2 expression is not induced in E26-transformed myeloblasts

Myeloid cells transformed by E26 represent a homogeneous population of immature myeloblasts that absolutely require cMGF for growth (Radke et al. 1982; Leutz et al. 1984). We investigated whether treatment of E26-transformed myeloblasts with cMGF also results in *c-ets-2* gene expression. E26-transformed myeloblasts were deprived of cMGF by incubation for 24 hr in cMGF-depleted growth medium and then restimulated with either cMGF or TPA for various periods of time; *c-ets-2* expression was analyzed by Northern blot hybridization. In contrast to observations in BMM, the expression of *c-ets-2* is barely detectable in either exponentially growing E26-transformed myeloblasts (data not shown) or in E26-transformed myeloblasts deprived of cMGF for 24 hr (Fig. 5). Furthermore, *c-ets-2* expression is not induced by either cMGF or TPA treatment of

Figure 5. Expression of *c-ets-2* in E26-transformed myeloblasts. RNA was extracted in guanidium isothiocyanate from a clone of E26-transformed myeloblasts either left untreated as a control or treated with either TPA or cMGF for 1, 4, 8, or 16 hr. After centrifugation, RNA was electrophoresed in agarose gels and analyzed by ethidium bromide staining (bottom; positions of the 28S and 18S rRNAs are marked at right) or, after transfer to nylon blot, by hybridization with *c-ets-2* cDNA probe (top).

Figure 6. (A and B) Subcellular localization of Ets2 in macrophages. Cells of the HD11 line were transferred to collagen-treated coverslips, fixed in 3.5% paraformaldehyde, treated with 0.15 M ethanolamine and permeabilized in 0.2% NP-40, and subcellular Ets2 localization was analyzed by immunofluorescence using an Ets2-specific peptide antiserum (A) or the Ets2-specific peptide antiserum blocked with an excess of its peptide immunogen (B). (C) DNA binding of Ets2 in vitro. Monkey COS-1 cells transfected with 10 μ g of PKCR3-Ck-3.1 were labeled with 250 μ Ci/ml L-[35 S]methionine, lysed, and incubated with native calf thymus DNA cellulose. Equivalent amounts of flowthrough (FT), wash (W), and elution (EI) fractions were immunoprecipitated with an Ets2-specific peptide antiserum and analyzed by SDS-polyacrylamide gel electrophoresis.

these cells over a 16-hr period. Similar results were obtained at the protein level (data not shown). These results suggest that the activation of *c-ets-2* in response to cMGF or PKC activators depends on the maturation state of the myelomonocytic cells where *c-ets-2* expression is rapidly and transiently activated in fully differentiated macrophages but not in immature cells of the myelomonocytic lineage.

Subcellular localization and DNA-binding activity of Ets2 products

We showed previously that Ets2 transiently expressed from the *c-ets-2* expression vector construct PKCR3-Ck-3.1 in monkey COS-1 cells is localized in the nucleus of these cells (Boulukos et al. 1988). In light of these results, we analyzed the subcellular localization of endogenous Ets2 in avian macrophages. Cells of the HD11 macrophage cell line were transferred to collagen-treated coverslips, and subcellular localization of Ets2 was analyzed by indirect immunofluorescence using the Ets2-specific peptide antiserum. As shown in Figure 6A, a high level of immunofluorescence is observed exclusively in the nuclei of HD11 cells when analyzed with the Ets2-specific peptide antiserum (Fig. 6A). This nuclear immunofluorescence signal is specific to Ets2 because it is not detected using either a nonimmune control serum (data not shown) or the Ets2 peptide antiserum blocked with an excess of the purified peptide immunogen (Fig. 6B).

We demonstrated recently that Ets1 displays DNA binding activity in vitro and that the Ca^{2+} -mediated phosphorylation of Ets1 results in a loss of this DNA binding capacity (Pognonec et al. 1989). Therefore, we

investigated whether Ets2 is also capable of binding to DNA in vitro. Monkey COS-1 cells transfected with PKCR3-Ck-3.1 were labeled with L-[³⁵S]methionine and lysed, and centrifuged lysates were incubated in the presence of native calf thymus DNA cellulose as described previously (Pognonec et al. 1989). Flowthrough, wash, and elution fractions were collected, and equivalent amounts of each were immunoprecipitated with an Ets2-specific peptide antiserum and analyzed by SDS-PAGE. As shown in Figure 6C, among the four products encoded by the *c-ets-2* cDNA, only the nonphosphorylated forms of 58 (p58) and 62 kD (p62) display affinity for DNA cellulose. This binding is specific to DNA, because no binding to plain cellulose is observed (data not shown). In contrast, the phosphorylated 60- (p60) and 64-kD (p64) forms are found exclusively in the flowthrough fraction. From these experiments, we conclude that Ets2, like Ets1, binds to DNA cellulose only in its nonphosphorylated state.

Discussion

In this paper we demonstrate that Ets2 is localized in the nuclei of chicken macrophages, where *c-ets-2* products are naturally and abundantly expressed and, like Ets1, only the nonphosphorylated Ets2 products of 58 and 62 kD are capable of binding to DNA in vitro. Ets1 and Ets2 products share two domains of identity of 96 and 175 amino acids near the amino terminus and at the carboxyl terminus, respectively (Boulukos et al. 1988). Therefore, it is likely that one or both of the conserved domains shared between Ets1 and Ets2 are required for DNA binding. Recent analyses of deletion mutants in these two conserved domains indicate that the DNA-binding domain appears to be the same for Ets1 and Ets2 and resides in the carboxyl terminus of these products (Boulukos et al. 1990).

We reported previously that Ets2 is expressed in normal and transformed chicken macrophages (Ghysdael et al. 1986b). In this paper we showed that expression of Ets2 in these cells is dependent on cMGF stimulation. The cMGF-induced expression of *c-ets-2* can be blunted by either down-regulation or inactivation of PKC, suggesting that PKC is involved in the intracellular cMGF signal transduction pathway in macrophages. Consistent with this, we found that Ets2 expression is also induced after treatment of these cells by TPA and LPS, both of which activate PKC. This induction is at least partially transcriptional because induction of *c-ets-2* expression is abolished in the presence of actinomycin D. In macrophages, Ets1 is weakly expressed and its expression is not induced by cMGF, TPA, or LPS, suggesting a distinct function of these two genes in these cells.

The induction of *c-ets-2* is transient in macrophages. We demonstrated that the half-life of *c-ets-2* RNA is short compared to most mammalian messages and can be lengthened by cycloheximide treatment. Sequence analysis of a chicken cDNA clone specific to Ets2 revealed at least three ATTTA motifs located in the 3'-

noncoding AT-rich region (K.E. Boulukos, unpubl.). AUUUA motifs are believed to destabilize mRNA and are characteristic of a number of transiently expressed genes, including *c-fos* and *c-myc* (for review, see Shaw and Kamen 1986). Taken together, these results indicate that *c-ets-2* is an early gene in its response to cMGF and PKC activators in fully differentiated cells of the myelomonocytic lineage.

Macrophages play an essential role in the inflammatory response and host defense mechanisms against infectious and neoplastic diseases. The cellular functions underlying these activities are acquired in the process of macrophage activation following exposure to external signals, including cytokines and bacterial cell wall products. One of the earliest events associated with macrophage activation is the rapid and transient induction or enhancement of a set of genes that are believed to contribute to the acquisition of functional competence (Hamilton et al. 1986; Introna et al. 1986, 1987; Tannenbaum et al. 1988). In this respect, *c-ets-2* resembles these genes in the rapid induction of its expression after LPS or TPA treatment of monocytes or macrophages. Unlike *c-fos* (Bravo et al. 1987), however, *c-ets-2* is not increased after treatment of BMM with the calcium ionophore A23187 or forskolin, an activator of adenylate cyclase (K.E. Boulukos et al., unpubl.). Because Ets2 products are nuclear and bind to DNA in vitro, the rapid and transient changes in their expression in response to LPS or cytokines may mediate in the nucleus some of the events contributing to the development of functional competence in macrophages.

In contrast to observations in nondividing, fully differentiated macrophages, *c-ets-2* gene expression is not induced by cMGF in E26-transformed myeloblasts. These results suggest that the response of macrophages and E26-transformed myeloblasts to cMGF is not the same. Why would cMGF activate *c-ets-2* gene expression in macrophages and not in E26-transformed myeloblasts? One possibility is that the expression of the E26-transforming protein (p135^{gag-myb-ets}) in E26-transformed myeloblasts interferes with the signal transduction pathway linking cMGF receptor occupation and *c-ets-2* gene expression. Coherent with this hypothesis, TPA treatment of E26-transformed myeloblasts also failed to result in the induction of *c-ets-2*.

However, cMGF clearly delivers a proliferative signal to E26-transformed myeloblasts because these cells are strictly dependent on cMGF for growth and survival (Leutz et al. 1984). Furthermore, we found that inactivation of p135^{gag-myb-ets} by a 24-hr shift to 42°C of myeloblasts transformed by a temperature-sensitive mutant of E26 in myeloid transformation (Beug et al. 1984) does not result in these cells responding to TPA by *c-ets-2* expression (J. Ghysdael, unpubl.). In fact, induction of *c-ets-2* expression after TPA treatment occurs only after 3 days of temperature shift when cells have acquired a more mature macrophage-like differentiation phenotype. It is therefore possible that signals elicited by cMGF to induce cell proliferation of immature cells of the myeloid lineage either are different or only partially

overlap with those mediating differentiated functions in mature cells. This differential signaling could result from the triggering of different cMGF receptors in immature and mature cells of the myeloid lineage or, alternatively, through the same receptor coupled to distinct signal transduction pathways. In any case, our results suggest that regulation of the expression of *c-ets-2*, at least in myelomonocytic lineage, depends on the maturation state of the cells and is not necessarily or solely linked to cell proliferation.

Materials and methods

Macrophage preparations and cells

Chicken BMM were obtained by culturing bone marrow cells for 4–6 days in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum and 2% chicken serum (standard medium) in the presence of 4 U/ml of cMGF (Leutz et al. 1984). Peritoneal macrophages from BALB/c mice were obtained as described essentially by Hamilton et al. (1986). Three days following intraperitoneal injection with 3% Brewer's thioglycollate broth, mice were sacrificed and macrophages were obtained after peritoneal washes with RPMI 1640 and calciparine. Peritoneal macrophages were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum for 24 hr before LPS induction analyses. The purification of human blood-derived monocytes was described previously (Sariban et al. 1987). Briefly, peripheral blood monocytes were isolated by Ficoll-Hypaque gradient sedimentation and adherence to plastic culture flasks. These adherent cells (activated monocytes) were incubated in RPMI 1640 medium containing 10% fetal bovine-defined serum for 24–48 hr before experiments were performed. The MC29-transformed HD11 and HBC-1 macrophage cell lines and myeloblast clone transformed by E26ts21 and freshly transformed AMV monoblasts used in this study were generously provided by H. Beug and T. Graf. E26-transformed myeloblasts and AMV-transformed monoblasts were maintained in standard medium in the presence of 1 U/ml cMGF (Leutz et al. 1984).

RNA preparation, cell labeling, and immunoprecipitation analyses

Total RNA was prepared from cells using guanidinium isothiocyanate. Ten micrograms of RNA was run on formaldehyde gels and transferred overnight to nylon membranes (Amersham). Probe preparation and washing of blots were performed as described previously (Boulukos et al. 1988).

Cells were labeled with L-[³⁵S]methionine, lysed in RIPA buffer [RIPA: 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 1% aprotinin (Sigma)] and immunoprecipitated as described previously (Ghysdael et al. 1986b). The standard *ets* antiserum used in this study was generated in rabbits, using a bacterially expressed protein corresponding to the carboxy-terminal domain of v-*ets* (Ghysdael et al. 1986a). The Ets2-specific peptide antiserum corresponding to the last 13 amino acids of the carboxyl terminus of Ets2 was obtained as described in Boulukos et al. (1988).

Immunofluorescence studies

Immunofluorescence analyses were performed as described previously (Boulukos et al. 1988). Briefly, cells of the macrophage

line HD11 were fixed in 3.5% paraformaldehyde 48 hr after transfection, treated with 0.15 M ethanolamine, and permeabilized in 0.2% NP-40. Cells were incubated with a control non-immune rabbit serum, an Ets2-specific peptide antiserum (Boulukos et al. 1988), or the Ets2-specific peptide antiserum blocked with an excess of its peptide immunogen. After washing, cells were incubated with FITC-conjugated swine anti-rabbit immunoglobulins (Dakopatts). Immunofluorescence was examined using a Zeiss epifluorescence microscope.

DNA-binding experiments

DNA-binding analyses were performed as described previously (Pognonec et al. 1989). Briefly, COS-1 cells were transfected with PKCR3-Ck-3.1 (Boulukos et al. 1988) and labeled with L-[³⁵S]methionine. Labeled cells were resuspended in TDT buffer [10 mM Tris-HCl (pH 7.4), 1 mM DTT, 0.1% Triton X-100, 1 mM EDTA, 10 µg/ml leupeptin, 100 µg/ml paramethylsulfonyl fluoride, and 1% aprotinin (Sigma)], homogenized, and nuclei pelleted at 1000g for 5 min. The supernatant was clarified by centrifugation at 100,000g and 4°C for 20 min. Nuclei were incubated for 10 min in 0.5 M NaCl–TDT buffer and centrifuged at 12,000g, and the nuclear extract was pooled with the first supernatant. After adjusting the final salt concentration to 50 mM NaCl, an aliquot of this preparation was loaded on double-stranded DNA or plain cellulose columns equilibrated in TDT buffer containing 50 mM NaCl. After incubation for 45 min, the flowthrough fraction was collected, the column was washed extensively in 50 mM NaCl–TDT, and finally eluted in TDT buffer containing 1M NaCl. Equivalent amounts of each fraction were adjusted to RIPA buffer conditions, immunoprecipitated, and analyzed by SDS-PAGE.

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