

EGR-1 prevents growth arrest by induction of *c-myc*

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Summary

The zinc-finger transcription factor EGR-1 provides protection from G1 phase growth arrest. We present here evidence that this protective effect of EGR-1 is linked to upregulation of *c-myc* RNA and protein by induction of the *c-myc* promoter. Growth arrest involves *c-myc* downregulation and hypophosphorylation of the retinoblastoma susceptibility protein RB, but upregulation of *c-myc* prevents hypophosphorylation of RB and provides protection from growth arrest. These findings suggest a downstream mechanism for EGR-1 function as an inhibitor of G1 phase growth arrest. Because *Egr-1* and *c-myc* are involved in determining cell fate in response to diverse exogenous signals, the findings of the present study can be extended to model systems for proliferation, cellular differentiation, and programmed cell death.

I. Introduction

Regulation of cell cycle progression by exogenous stimuli is associated with a series of complex molecular cascades that are initiated at the plasma membrane and are dependent on inducer- and cell type-specific gene programs. Gene programs in the G1 phase of the cell cycle control entry into, or exit from, the cell cycle and thereby dictate the cell's ultimate responses to exogenous stimuli. Thus, these gene programs couple the biochemical signaling events occurring at the plasma membrane to long-term alterations in cellular phenotypes such as proliferation, growth arrest, differentiation, and programmed cell death. G1 phase genes that encode transcription factors are key regulators of the downstream cascades that determine entry past the G1 phase cell cycle check point into the S-phase. Understanding the role of such transcription factors in the positive and negative regulation of growth will provide important insights into how extracellular stimuli signal specific long-term cellular responses.

The transcription factor early growth response-1 (EGR-1; also cited as NGF-1A, TIS8, Zif268, and Krox24), encoded by the *Egr-1* gene, is upregulated in response to diverse cellular stimuli including mitogens, membrane depolarization, seizure, synaptic activity, ischemia, nerve

injury, and B-cell maturation and differentiation of nerve, bone and myeloid cells (Milbrandt, 1988; Sukhatme, 1988). EGR-1 is a nuclear protein that contains three zinc-fingers of the C₂H₂ subtype (Cao et al., 1990; Christy and Nathans, 1989; Gashler et al., 1993; Swirnow and Milbrandt, 1995). Deletion analysis of the *Egr-1* cDNA has shown that the amino acids constituting the zinc-finger domain and the flanking regions confer DNA-binding and nuclear localization functions (Gashler et al., 1993; Russo et al., 1993). The amino-terminus contains a serine/threonine-rich region that confers a transcription activation function (Gashler et al., 1993; Russo et al., 1993). EGR-1 is the prototypic member of the Egr family of transcription factors that includes EGR-2/Krox-20, EGR-3, NGFI-C, and the gene product of the Wilms' tumor suppressor gene, WT1 (Call et al., 1990; Crosby et al., 1991; Lau and Nathans, 1987; Lemaire et al., 1988; Lim et al., 1987; Sukhatme et al., 1988; Sukhatme, 1990). The transcription factors of the Egr family have a high degree of homology in the amino acid sequence constituting the zinc-finger region, and they bind to the same GC-rich consensus DNA sequence (Christy and Nathans, 1989; Rauscher et al., 1990; Sukhatme, 1990). The direct interaction between the GC-rich consensus DNA elements and the zinc-finger domain of EGR-1 has been confirmed by X-ray crystallography studies (Pavletich and

Pabo, 1990). Transient transfection studies using EGR-1 or WT1 expression vectors and reporter genes that contain GC-rich consensus element have shown that these proteins can function either as strong activators or as repressors of transcription, depending upon the cellular context (Drummond et al., 1992; Madden et al., 1991; Maheswaran et al., 1993; Wang et al., 1992).

Consistent with the fact that EGR-1 is induced in response to a wide spectrum of mitogenic stimuli, GC-rich EGR-1-binding sites have been identified in the promoters of genes such as thymidine kinase, an enzyme integral to DNA biosynthesis (Molnar et al., 1994); in cell cycle regulators such as cyclin D1 (Phillipp et al., 1994) and in the gene encoding the retinoblastoma susceptibility protein RB involved in cellular proliferation (Day et al., 1993). Recent studies, however, suggest that DNA sequences that diverge from the consensus sequence can also bind EGR-1 with high affinity (Molnar et al., 1994; Swirnoff and Milbrandt, 1995). DNA-protein binding studies *in vitro* have shown that S1-nuclease sensitive regions, which are homopurine/homopyrimidine-rich [(TCC)_n] in nucleotide content, can bind EGR-1 just as avidly as can the GC-rich consensus element (Wang and Deuel, 1992; Wang and Deuel, 1996). Such homopurine/homopyrimidine-rich motifs have been identified in the promoter regions of several genes encoding growth factors such as platelet-derived growth factor, transforming growth factor β (TGF β) and basic fibroblast growth factor; growth factor receptors epidermal growth factor-receptor, and the insulin-like growth factor-receptor; and protooncogenes *c-Ki-ras* and *c-myc* (Gashler and Sukhatme, 1995; Hu and Levin, 1994). However, the downstream phenotypic consequences of EGR-1-binding to the homopurine/homopyrimidine-rich motifs in these gene promoters are not known.

c-MYC, the product of the *c-myc* gene is another G1 phase transcription factor that is induced in response to mitogenic stimuli and essential for cell-cycle progression (reviewed in Luscher and Eisenman, 1990). The c-MYC protein contains a basic region-helix-loop-helix domain and a leucine zipper domain at its carboxy-terminus for the DNA-binding function (Blackwell et al., 1993). The target DNA-binding motif for MYC is a 6-bp consensus sequence 5'-CTCGAG-3' referred to as the E-box (Blackwell et al., 1993). The transcription activation function of c-MYC is conferred by the presence at its amino-terminus of a proline/glutamine-rich region and of flanking amino acid residues (Blackwell et al., 1993). Several studies have implicated c-MYC in the regulation of proliferation, programmed cell growth arrest, differentiation, or apoptosis (Alexandrow et al., 1995; Cole, 1986; Evan and Littlewood, 1993; Hanson et al., 1994; Hermeking and Eick, 1994; Hoffman-Liebermann and Liebermann, 1991; Janicke et al., 1994; Janicke et al., 1996; Luscher and Eisenman, 1990; Wagner et al., 1994). Inhibiting *c-myc* expression by using antisense oligonucleotides causes growth inhibition of proliferating cells (Evan and Littlewood, 1993). Consistent

with this observation, ectopic overexpression of *c-myc* in quiescent cell cultures is sufficient to induce the cells to re-enter the cell cycle, even in the absence of serum growth factors (Evan and Littlewood, 1993). Moreover, *c-myc* overexpression can protect cells from the growth arresting action of TGF β (Alexandrow et al., 1995). In cells that can be induced to differentiate, enforced expression of *c-myc* prevents the cells from exiting the cell cycle and thereby inhibits the differentiation pathway (Hoffman-Liebermann and Liebermann, 1991). In other studies, cells that are deprived of serum growth factors have been shown to undergo apoptosis when *c-myc* is ectopically overexpressed (Evan and Littlewood, 1993; Hartwell and Kastan, 1994). Collectively, these studies have firmly established *c-myc* as a regulator of diverse long-term cellular responses.

Although c-MYC plays a central role in the regulation of proliferation, differentiation, and apoptosis, the precise mechanism by which c-MYC regulates these long-term growth responses is not known. Several examples of differential regulation of growth-associated genes by c-MYC have been presented. Enforced expression of *c-myc* constitutively activates the expression of ornithine decarboxylase, an enzyme integral to polyamine biosynthesis and a mediator of apoptosis after IL-3 withdrawal in murine myeloid cells (Packham and Cleveland, 1994). Constitutive expression of *c-myc* can transactivate the expression of the cell cycle regulators cyclin D1, D3, and E (Janicke et al., 1996; Janson-Durr et al., 1993) or cyclin A (Janson-Durr et al., 1993). Other studies have shown that in different cell types, constitutive expression of *c-myc* can transcriptionally regulate the expression of cyclin-dependent kinase 4 (cdk4), a key regulator of cell cycle progression in the G1 phase (Hanson et al., 1994). It is also suggested that *c-myc* can function as a "molecular matchmaker", a factor that can alter the affinities of two or more interacting factors that mediate cell cycle progression, in a manner independent of transcriptional activation (Hanson et al., 1994). Examples of this activity include the ability of c-MYC to interfere with the interaction between the retinoblastoma susceptibility gene product RB and E2F, and the ability to promote increased complex formation between cyclin A, cyclin dependent kinase 2 and the transcription factor E2F (Marcu et al., 1992; Hanson et al., 1994; Janson-Durr et al., 1993). The formation of these complexes regulates the G1 to S phase transition of the cell cycle (Hartwell and Kastan, 1994; Li et al., 1993; Pietenpol et al., 1990; Qin et al., 1995; Weinberg, 1995). Finally, recent evidence suggests that c-MYC can modulate the activity of cdk4 by transcriptional regulation of *cdc25* which encodes a phosphatase that directly controls the activity of cdk4 (Galaktionov et al., 1996). All of these examples represent viable, biologically-relevant mechanisms for c-MYC function; however, the mechanisms are cell type-specific, suggesting multiple mechanisms for cell growth control by c-MYC.

Our studies have focused on the role of immediate-early genes in programmed cell growth arrest (Rangnekar et al., 1991; Rangnekar et al., 1992). These studies have used, as an experimental model, human melanoma cells A375-C6 (Endo et al., 1988) that are susceptible to cytokines such as interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α) (Rangnekar et al., 1991; Rangnekar et al., 1992). We have demonstrated that in A375-C6 cells, IL-1 induces a G1 phase growth arrest response that is dependent on hypophosphorylation of the retinoblastoma susceptibility protein RB (Muthukkumar et al., 1996). IL-1 causes induction of EGR-1 in A375-C6 cells (Sells et al., 1995), and our initial studies showed that this protein functions to prevent RB hypophosphorylation (Krishnan and Rangnekar, unpublished data) and to protect the cells from growth arrest (Sells et al., 1995). We then sought to determine the mechanism by which EGR-1 may confer this protective effect. We present here evidence that EGR-1 up-regulates *c-myc* expression by inducing its promoter and thereby protects A375-C6 cells from RB-hypophosphorylation and IL-1-inducible growth arrest.

II. Materials and Methods

A. Cell culture and cytokine

Growth and maintenance of human melanoma cells A375-C6 have been described previously (Rangnekar et al., 1991; Rangnekar et al., 1992). Human recombinant IL-1 β (specific activity, 1.8×10^7 units/mg) was a gift from Craig Reynolds, Biological Response Modifiers Program, National Cancer Institute (Fredrick, MD). IL-1 was used at a concentration of 100 unit/ml as previously described (Rangnekar et al., 1991; Rangnekar et al., 1992).

B. Plasmid constructs

Previous studies have described the cytomegalovirus (CMV) promoter-based expression constructs for mouse-EGR-1 (CMV-mEGR1); the EGR-1-WT1 chimera (CMV-WT1/EGR1) that contains the transactivation domain of WT1 and the three zinc finger domains of EGR-1; and deletion mutants of EGR-1 (from Vikas P. Sukhatme, Harvard Medical School, Boston, MA) (Drummond et al., 1992; Gashler et al., 1993; Madden et al., 1991; Nair et al., 1997). The pAc.myc construct (Hoffman-Liebermann and Liebermann, 1991), which contains a 1.5 kb Sst1-HindIII fragment with 60 bp of untranslated region and all of the coding region (exons 2 and 3) of murine *c-myc* cDNA cloned into the HindIII site of the actin-promoter construct, pHb Apr-1-neo, was kindly provided by Barbara Hoffman (Temple University School of Medicine, Philadelphia, PA). The reporter construct myc(-1141+513)CAT that contains 1141 bp upstream and 513 bp downstream of the P1 promoter of murine *c-myc* linked to chloramphenicol acetyl transferase (CAT) cDNA was provided by John Cleveland,

(Saint Jude Children's Hospital, Memphis, TN); and [myc(E-Box)CAT] that contains the c-MYC response element upstream of CAT cDNA, was provided by Robert Eisenman (Fred Hutchinson Cancer Center, Seattle, WA). The CAT reporter constructs myc(-665+935)CAT and myc(-101+935)CAT, which contain 665 bp and 101 bp upstream, respectively, and 935 bp downstream of the P1 promoter of human *c-myc* (Wang and Deuel, 1992), were kindly provided by Zhao-Yi Wang (Harvard Medical School, Boston, MA).

To generate the reporter constructs p90-CAT, p513-CAT, or p579-CAT, polymerase chain reaction (PCR) was used to amplify various fragments containing the different regions of the human *c-myc* promoter region. The p579 and p90 fragments used a common downstream primer 5'-(CTCGAT)TGCTTTGGGA-3' (which contains 10 nucleotides (underscored) complimentary to nucleotides -96 to -86 upstream of *c-myc* P1 promoter, and a built-in XhoI site shown in parentheses). For construct p579, a 579 bp fragment containing nucleotides -665 and -86 from the upstream region of human *c-myc* was synthesized using primer 5'-ATACATGACTCCCCCAACA-3' and the common downstream primer. For construct p90, a 90 bp fragment containing nucleotides -176 and -86 from the upstream region of human *c-myc* was synthesized by using primer 5'-(GTTCGAC)AGAGTGCTCGGC-3' (Sal I site in parentheses) and the common downstream primer. For construct p513, a 513 bp fragment containing nucleotides -685 and -1528 from the upstream region of *c-myc* (i.e., representing a deletion of the -145 to -115 homopurine/homopyrimidine region) was generated by using the same upstream primer as that used for the 579 bp fragment, and a downstream primer, 5'-ACTCAGCCCGGGCAGCCGAGCACT-3'. Each of the three fragments were subcloned into the SmaI site of pBluescript II SK(-) (Stratagene, La Jolla, CA) and fidelity of the fragments verified by sequencing. Further amplification of the three fragments was accomplished by PCR by using the proof-reading DNA polymerase Pfu (Stratagene), and the primers T3 (5'-AATTAACCCTCACTAAAGGG-3') and T7 (5'-GTAATACGACTCACTATAGGGC-3'). The amplified, blunt-ended fragments were then subcloned into the BglII site of pCAT (Promega Corp., Inc., Madison, WI) after filling-in the 3' overhangs of the BglII site with Klenow fragment. The pCAT vector contains a minimal SV-40 early promoter driving the CAT cDNA. The orientation of the insert and the fidelity of the final constructs were confirmed by sequencing.

C. Northern analysis

Total RNA preparation and Northern blot analysis was performed as described (Muthukkumar et al., 1995; Sells et al., 1995). The EcoRI/HindIII fragment of pMV-7/mycER which contains exons 2 and 3 of human *c-myc* was used as a

probe for *c-myc*. The cDNA probe for human *gro-β* has been described (Rangnekar et al., 1991). To verify equal loading of RNA on the gel, the blots were probed with cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The hybridization signals were scanned with a densitometer to measure the amount of mRNA, and the amount of fold induction or reduction was calculated after normalizing the hybridization signal with respect to GAPDH.

D. Assay for ³[H]thymidine incorporation

These studies were performed in 96-well plates and percent growth inhibition was calculated as described previously (Rangnekar et al., 1991; Rangnekar et al., 1992).

E. Effect of IL-1 on spheroid growth

Cells were plated in plates coated with semi-solid growth medium (RPMI 1640 growth medium containing 4% gelatin) with a top layer of the liquid growth medium. When cells began to clump and form spheroids, individual spheroids of similar size and shape were transferred to a 24-well plate. The spheroids were then either exposed or left unexposed to IL-1. The dimensions of each spheroid were determined daily by microscopy with a scaled grid and the volume (width² X length) was calculated.

F. DNA transfections and stable transfectant cell line

Transfections were performed by using calcium phosphate coprecipitation method described previously (Sells et al., 1995). Stable transfectant clones were selected by culture in 300 μg/ml G418 sulfate (BRL/Life Technologies, Inc.). Pools of G418-resistant clones were maintained as cell lines.

G. CAT assays

Transient transfections and CAT assays were performed as previously described (Sells et al., 1995). The values for percent conversion of [¹⁴C]chloramphenicol to acetylated forms in different samples from a given experiment were normalized with respect to the corresponding protein concentration and were expressed as relative CAT activity.

H. Western (immunoblot) analysis

Whole-cell protein extracts were subjected to Western blot analysis with the indicated antibody (1 mg/ml) and ¹²⁵I-protein A, as described previously (Ahmed et al., 1996; Muthukkumar et al., 1995). The retinoblastoma susceptibility gene product RB was detected by using the anti-RB antibody C-15 (from Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (Muthukkumar et

al., 1996). The anti-β-actin monoclonal antibody was purchased from Sigma Chemical Company (St. Louis, MO). Blots that were first probed with the monoclonal antibodies were subsequently probed with a rabbit anti-mouse antibody (from Southern Biotechnology, Birmingham, AL) before incubation with ¹²⁵I-protein A. The rabbit polyclonal antibody for MYC, 50-39 was a generous gift from Steve Hann (Vanderbilt University, Nashville, TN). Western blot analysis for the detection of MYC was carried out as described above, except that after incubation with the MYC antibody, the Enhanced ChemoLuminescence (ECL; Amersham, Arlington Heights, IL) system was used for detection.

III. Results

A. Ectopic expression of EGR-1 protects cells from growth arrest

We have previously reported that EGR-1 mRNA and protein are induced in A375-C6 cells after 3 to 4 hours of exposure to IL-1, and that inhibition of EGR-1 expression or function enhances the growth-inhibitory effect of IL-1 (Sells et al., 1995). We sought to examine whether ectopic overexpression of EGR-1 can protect A375-C6 cells from IL-1-inducible growth arrest. These experiments used constructs that encoded either full-length EGR-1 or functional variants of EGR-1 such as those that lacked the transactivation region (ΔTA) or DNA-binding region (ΔZF). A375-C6 cells were stably transfected with the different constructs and pools of stably transfected clones were maintained as cell lines. The transfected cell lines were exposed to IL-1 and growth inhibition was determined by [³H]thymidine incorporation assays. As shown in **Fig. 1** (A & B), ectopic expression of full-length EGR-1 from CMV-mEGR1 caused a significant decrease ($P < 0.0001$ by the Students *t* test) in growth inhibition relative to cells containing the empty vector after exposure to IL-1. By contrast, cells expressing either the EGR-1-mutant ΔZF that was deficient in DNA-binding or ΔTA that was deficient in transcriptional activity were similar to those containing the empty-vector in terms of their susceptibility to IL-1-inducible growth arrest (**Fig. 1B**). These findings suggest that EGR-1 confers protection from a growth arrest signal and that the protective effect is dependent on the ability of EGR-1 to transcriptionally regulate downstream genes.

B. EGR-1 induces the *c-myc* promoter

In the course of our studies aimed at identifying downstream targets of EGR-1, we tested *c-myc* because its promoter (P1) contains an EGR-1-binding element with a homopurine/homopyrimidine-rich sequence (Wang and

Figure 1. Effect of ectopic overexpression of EGR-1 or deletion mutants on IL-1-inducible growth arrest. (A) Pools (L1 or L2) of stably transfected clones expressing empty vector or EGR-1 were exposed to IL-1 or vehicle for 24, 48, or 72 hours. (B) Pools (L1) of stably transfected clones expressing empty vector, EGR-1, EGR-1-transactivation deletion mutant Δ TA, or EGR-1-zinc finger deletion mutant Δ ZF were exposed to IL-1 or vehicle for 72 hours. The cells were subjected to [3 H]thymidine incorporation studies and percent growth inhibition was calculated. Each value point is a mean of 12 observations from 3 different experiments. Error bars indicate *standard deviations.

Deuel, 1992) [(TCC)_n located -142 to -115 bp; depicted in **Fig. 2**]. This element has been previously shown to bind, in electrophoretic mobility shift assays, to purified EGR-1 (Wang and Deuel, 1992). However, the ability of this element to induce EGR-1-dependent transactivation of the *c-myc* promoter has not been tested. Secondly, significant overlap in EGR-1 and *c-myc* functions has been identified: (i) EGR-1 and c-MYC are both associated with induction of proliferation in most cell types (Evan and Littlewood, 1993; Gashler and Sukhatme, 1995); (ii) forced expression of *Egr-1* or *c-myc* can block or restrict differentiation along a specific cellular lineage (Hoffman-Liebermann and Liebermann, 1991; Nguyen et al., 1993); and (iii) *Egr-1* or *c-myc* expression sensitizes cells to apoptotic stimuli (Ahmed et al., 1996; Hermeking and Eick, 1994; Janicke et al., 1994; Sells et al., 1995; Wagner et al., 1994). To determine the ability of EGR-1 to transcriptionally activate the promoter of

c-myc, we used CAT-reporter constructs containing various deletions of the P1 promoter of *c-myc*. Cells were cotransfected with CMV-mEGR1, or as controls with an empty vector or CMV-WT1/EGR1, and the CAT-reporter constructs. The CMV-WT1/EGR1 construct was expected to transrepress the promoter constructs that were transactivated by CMV-mEGR1.

When a constant amount of the CAT-reporter construct [myc(-1141+513)CAT] which contains 1141 bp upstream or 513 bp downstream of the *c-myc* promoter (**Fig. 2A**) was cotransfected with increasing amounts of CMV-mEGR1, a 5 to 6 fold increase in CAT activity occurred (**Fig 2B**). On the other hand, cotransfection with CMV-WT1/EGR1 caused a decrease in CAT activity from the [myc(-1141+513)CAT] construct (**Fig. 2B**). These findings suggest that ectopic expression of EGR-1 leads to transcriptional upregulation of the *c-myc* promoter.

Figure 2.
Transactivation of the c-myc promoter deletion constructs by EGR-1. (A) Schematic of myc(-1141+513)CAT, myc(-665+935)CAT, myc(-101+935)CAT reporter constructs used for transfections. The cap site and the (TCC)_n region are indicated. A375-C6 cells were cotransfected (B) reporter plasmid myc(-1141+513)CAT (4 μ g) and with various amounts of CMV-mEGR1 or CMV-WT1/EGR1; with (C) myc(-1141+513)CAT (4 μ g) and 5 μ g of vector, CMV-mEGR1, CMV-mEGR1. Δ TA, or CMV-mEGR1. Δ ZF; or with (D) myc(-665+935)CAT or myc(-101+935)CAT reporter constructs and various amounts of CMV-mEGR1. The total amount of plasmid DNA used in each transfection was brought up to 34 μ g by using vector DNA. Cell extracts were assayed for CAT activity and relative CAT activity with each effector construct is expressed relative to that with vector 30 μ g of empty vector.

We then examined whether induction of the *c-myc* promoter required the DNA-binding and transactivation functions of EGR-1. Cells were transiently cotransfected with vector, CMV-mEGR1, CMV-mEGR1. Δ ZF, or CMV-mEGR1. Δ TA and the reporter plasmid myc(-1141+513)CAT, and cell lysates were examined for CAT activity. As seen in **Fig. 2C**, CMV-mEGR1 induced the *c-myc* promoter. On the other hand, the EGR-1 mutants CMV-mEGR1. Δ ZF or CMV-mEGR1. Δ TA did not induce the promoter (**Fig. 2C**). These findings suggest that the DNA-

binding and transactivation functions of EGR-1 are both required for induction of the *c-myc* promoter.

To further localize the EGR-1-responsive site(s) within the *c-myc* promoter, transient transfections were performed by using the reporter construct myc(-665+935)CAT, which is a truncated variant of myc(-1141+513)CAT and contains 665 bp of sequence upstream of P1 promoter (**Fig. 2A**). When cotransfected with CMV-mEGR1, myc(-665+935)CAT showed a 2 to 3 fold increase in CAT activity (**Fig. 2D**). Cotransfection of CMV-WT1/EGR1 caused a dose-dependent decrease in CAT activity from this same

construct (data not shown). These results suggest that the EGR-1-responsive element is contained within 665 bp upstream and 995 bp downstream of the *c-myc* promoter.

When reporter construct myc(-101+935)CAT containing 101 bp upstream and 935 bp downstream of the promoter [i.e., lacking the -142 to -115 homopurine/ homopyrimidine-rich region and upstream sequence] was cotransfected with CMV-mEGR1 (**Fig. 2D**) or CMV-WT1/EGR1 (data not shown) neither an increase nor a decrease in CAT activity was detected. The lack of response of this reporter construct to EGR-1 or the WT1/EGR-1 chimera suggests that the putative EGR-1 response element is located in the region between -665 and -101 bp of the *c-myc* promoter.

C. EGR-1 regulates *c-myc* expression via the (TCC)_n EGR-1-binding motif in the *c-myc* promoter

We next tested the (TCC)_n motif located between 142 to 115 upstream of P1 promoter for inducibility with EGR-1. Cells were cotransfected with CMV-mEGR1 and the following reporter constructs (**Fig. 3A**): p90-CAT which contains the (TCC)_n region; construct p513-CAT in which the (TCC)_n region is absent; or p579-CAT which contains the region -665 to -86 and was expected to be an EGR-1-responsive control (based on results from **Fig. 2D**), and CAT assays were then performed. CMV-mEGR1 caused a 3 to 3.5 fold induction in CAT activity from p90-CAT or p579-CAT but did not alter the basal CAT activity from p513-CAT, (**Fig. 3B**). These findings suggest that EGR-1 causes induction of the *c-myc* promoter via the (TCC)_n motif.

Figure 3. Transactivation of *c-myc* promoter via the (TCC)_n sequence by EGR-1. (A) Schematics of pSV40-CAT, p579-CAT, p90-CAT and p513-CAT. These constructs used the SV-40 promoter and various fragments corresponding to the *c-myc* promoter region. The (TCC)_n region and the cap site are indicated. Note that p513-CAT lacks the (TCC)_n motif. (B) The reporter plasmids (10 μg) were cotransfected with 0 or 10 μg of vector or CMV-mEGR1 and cell extracts were assayed for CAT activity. Relative CAT activity for each reporter construct is a ratio of the CAT activity with 10 μg of CMV-mEGR1 and 10 μg of empty vector.

Figure 4. EGR-1 induces c-myc expression at the mRNA and protein level. Total RNA isolated from C6/EGR1.L1 or C6/vector.L1 cells was subjected to Northern blot analysis for c-myc or GAPDH. The increase in induction of c-myc RNA normalized to GAPDH expression levels is indicated. **(B)** Whole-cell extracts from C6/vector.L1, C6/EGR1.L1, C6/myc.L1 or C6/myc.L3 cells were prepared and subjected to Western blot analysis by using the anti-MYC antibody, 50-39. For a loading control, the same blot was probed with anti- β actin antibody. The arrow indicates the position of MYC protein (about 68 kd).

D. Overexpression of EGR-1 causes increased expression of c-myc RNA and protein

Our results suggested that the c-myc promoter represented one of the targets of EGR-1. We then ascertained that this effect was not restricted to the promoter and that it resulted in an increase in c-myc RNA and protein. Total RNA was isolated from cell lines that were stably transfected with CMV-mEGR1 or vector and was subjected to Northern blot analysis for c-myc. Data representative of four different pools of transfected clones maintained as cell lines are shown in **Fig. 4**. As compared to C6/vector cells, C6/EGR1 cells showed a 5 fold increase in c-myc RNA (**Fig. 4A**). To determine whether the protein levels of c-MYC were also higher in cells overexpressing EGR-1, we prepared whole-cell protein extracts from the transfectants and subjected them to Western blot analysis for c-MYC protein. As seen in **Fig. 4B**, C6/EGR1.L1 cells express a 4 fold higher level of c-MYC protein than do C6/vector.L1 cells. These results suggest that EGR-1 can upregulate endogenous c-myc, leading to increased levels of c-MYC protein.

E. IL-1 mediated growth arrest is dependent on downregulation of c-myc RNA in A375-C6 cells

To study the biological relevance of EGR-1-inducible expression of c-myc, we used A375-C6 cells that undergo a time- and dose-dependent growth arrest in the G1 phase of

the cell cycle when exposed to IL-1. Because the levels of c-myc expression correlate with the growth status of various cell lines, we began these studies by examining the effect of IL-1 on c-myc expression in A375-C6 cells. Total RNA was isolated from subconfluent monolayers of A375-C6 cells before or after exposure to IL-1 and was examined for c-myc expression by Northern analysis. As seen in **Fig. 5A**, within 1 hour of exposure to IL-1, c-myc levels decreased by 8 fold relative to the basal levels; thereafter, sustained low levels of c-myc were maintained upto 48 h of exposure to IL-1. When the same Northern blot was probed with gro- β cDNA, a rapid and sustained induction of the gro- β gene was seen indicating responsiveness to IL-1 as expected from our previous studies (Joshi-Barve et al., 1993; Rangnekar et al., 1991; Rangnekar et al., 1992).

We also ascertained that the c-myc gene in A375-C6 cells is positively or negatively inducible by growth-stimulatory or growth-inhibitory signals, respectively. The cells were grown in serum-containing medium (unstarved cells) or serum-free medium (serum-starved cells) for 48 h and were then exposed to 10% serum for various intervals of time. Total RNA was prepared and subjected to Northern blot analysis for c-myc. As seen in **Fig. 5B**, serum-starvation caused a rapid decrease in the steady state levels of c-myc, and serum-stimulation caused a strong induction of c-myc within 2 to 3 hours. These findings suggest that the expression levels of c-myc in these cells are positively regulated by growth-stimulatory signals and are negatively regulated by growth arresting signals.

Figure 5. IL-1-inducible growth arrest is associated with downregulation of *c-myc* mRNA. (A) A375-C6 cells were treated with vehicle (untreated with IL-1 [UT]) or were treated with IL-1 for various intervals of time, as indicated. Total RNA was then prepared and subjected to Northern analysis for *c-myc*. The same blot was sequentially probed for *gro-β*, and finally for GAPDH expression. (B) A375-C6 cells were grown in medium containing 10% serum (unstarved [US]), or were serum-starved for 48 hours (0 h of serum-stimulation) and were then exposed to 10% serum. Total RNA was isolated at various time points after serum restimulation and was subjected to Northern blot analysis for *c-myc* and GAPDH.

F. Ectopic overexpression of *c-myc* protects A375-C6 cells from IL-1–inducible growth inhibition.

To determine whether downregulation of *c-myc* is required for the growth-inhibitory action of IL-1 in A375-C6 cells, we studied whether ectopic overexpression of *c-myc* driven by the β -actin promoter (which was expected to be unresponsive to IL-1) could alter the growth-inhibitory response. A375-C6 cells were stably transfected with pAc.myc, an eukaryotic expression vector in which exons 2 and 3 of *c-myc* are under the transcriptional control of the β -actin promoter. Pools of stably transfected clones were maintained as cell lines and were subjected to Northern blot analysis to verify expression of the 1.8 kb RNA from the transgene and to Western blot analysis to verify increased c-MYC protein levels. As seen in **Fig 6A**, several G418-resistant clones (L1 and L3, but not L2) expressed high levels of the 1.8 kb transgenic *c-myc* RNA species and of the 2.4 kb RNA species representing endogenous *c-myc*. Western blot analysis indicated that myc.L1 and myc.L3 cells that expressed the transgenic *c-myc* RNA showed an higher amount of c-MYC protein than did cells transfected with vector alone (**Fig. 4B**). A [³H]thymidine incorporation assay performed to study the growth rate of the different cell lines indicated that the doubling times for all of the C6/myc and C6/vector transfected cell lines examined over a 72 h period were similar (i.e., about 24 h; data not shown). Finally, to ascertain whether the transgenic c-MYC protein was functional, we used transient transfection of the myc.L1 or vector.L1 cultures with a CAT construct containing the MYC-responsive E-box sequence. As seen in **Fig. 6B**, myc.L1 cells showed a 3 to 4 fold higher CAT activity than

did the vector.L1 cells. Together, the findings of these studies indicated that transgenic *c-myc* is both expressed and functional in A375-C6 transfectants.

action of IL-1. Stable transfectants expressing *c-myc* or vector were grown at maximum density on a non-adherent culture surface to obtain spheroids. Individual spheroids were transferred to a 24-well culture plate and then either left unexposed or exposed to IL-1, and the increase in spheroid volume was determined over a 11 day period. The findings from these experiments (**Fig. 7B**) indicated that IL-1 caused a 30 to 45% growth inhibition of spheroids from vector-transfected cells, and a 10 to 15% growth inhibition of spheroids from *myc*-transfected cells. These findings are consistent with those from the [³H]thymidine incorporation studies and suggest that ectopic expression of *c-myc* protects A375-C6 cells from the growth-inhibitory action of IL-1.

Figure 6. Expression of *c-myc* in stably transfected clones.

(A) Total RNA was prepared from A375-C6 cells that were stably transfected with vector or *c-myc* expression plasmid and Northern blot analysis was performed by using *c-myc* cDNA as a probe. Note that C6/*myc*.L1 and C6/*myc*.L3 expresses both the endogenous *c-myc* 2.4 kb RNA and the transgenic *c-myc* 1.8 kb RNA; whereas C6/vector.L1 and C6/*myc*.L2 express only endogenous *c-myc* RNA. (B) Transfected cell lines C6/vector.L1 or C6/*myc*.L1 were transiently transfected with MYC(E-box)-CAT reporter plasmid which contained the MYC-response element upstream of the CAT cDNA or with min-CAT plasmid, which contained a minimal promoter but lacked the E-box. The cell extracts were then assayed for CAT activity.

To determine whether ectopic expression of *c-myc* could rescue the cells from growth arrest by IL-1, C6/vector or C6/*myc* cell lines were left unexposed or exposed to IL-1 for 24, 48, or 72 h, and the effect on growth was examined by [³H]thymidine incorporation studies. The vector-transfected cell lines showed approximately 25, 40, or 70% growth inhibition in response to IL-1 at 24, 48, or 72 h, respectively (**Fig. 7A**). By contrast, the C6/*myc* cell lines showed a significant decrease ($P < 0.0001$ by the Student *t* test) in susceptibility to IL-1, with a maximum of approximately 50% growth inhibition after 72 hours exposure to IL-1 (**Fig. 7A**).

We also examined whether ectopic expression of *c-myc* rescued cells grown as spheroids from the growth arresting

G. Ectopic overexpression of *c-myc* prevents hypophosphorylation of RB

The retinoblastoma susceptibility gene product RB is a key regulator of the G1 phase growth arrest action of IL-1 in A375-C6 cells (Muthukkumar et al., 1996). Exposure to IL-1 causes hypophosphorylation of RB protein that is functionally required for growth arrest (Muthukkumar et al., 1996). Because ectopic expression of *c-myc* protects the cells from IL-1-inducible growth arrest, we sought to determine whether this protective mechanism was linked to the phosphorylation status of RB. The C6/vector.L1 or C6/*myc*.L1 cell lines were left unexposed or exposed to IL-1 for 48 h and protein extracts were subjected to Western blot analysis. As seen in **Fig. 7C**, untreated C6/vector. L1 cells contained about 10% of total RB in the hypophosphorylated form, and treatment with IL-1 caused an accumulation of the faster-migrating, hypophosphorylated form of RB with a concomitant decrease (about 50%) in the slower-migrating, hyperphosphorylated form of RB. By contrast, there was only a minimal change in the phosphorylation status of RB in the C6/*myc*.L1 cells exposed to IL-1, with <15% hypophosphorylated RB accumulating after 48 h (**Fig. 7C**). These findings indicate that ectopic expression of *c-myc* abrogates the IL-1-inducible events that lead to hypophosphorylation of RB.

Figure 7. The effect of *c-myc* overexpression on IL-1-inducible growth inhibition and hypophosphorylation of RB. (A) C6/myc.L1 or vector.L1 cultures were exposed to IL-1 or vehicle for 24, 48, and 72 h, and then subjected to [³H]thymidine incorporation studies. Each value point is a mean of 12 observations from 3 different experiments. Error bars indicate \pm standard deviations. (B) Spheroids produced from C6/myc.L1 or C6/vector.L1 cultures were exposed to IL-1 or vehicle, and 3-dimensional growth was determined at the various time points indicated. Each value point is a mean of 24 observations from 3 separate experiments. (C) C6/myc.L1 or C6/vector.L1 transfected cells were unexposed (-) or exposed (+) to IL-1 for 48 hours, and then whole-cell protein extracts were prepared from the cells and subjected to Western blot analysis using the anti-RB antibody. The slow-migrating differentially phosphorylated forms of RB (pRB) and the fast-migrating hypophosphorylated form of RB (RB) are indicated.

IV. Discussion

The present study used an *in vitro* growth arrest system to determine the effect of EGR-1 expression on a G1 phase growth arrest pathway. We determined that EGR-1 functions to protect A375-C6 cells from the growth arresting action of IL-1. Furthermore, this study demonstrated that EGR-1 can regulate the expression of the *c-myc* gene via a (TCC)_n EGR-1-binding element. EGR-1 has been previously shown to directly bind to this element (Wang and Deuel, 1992), and our present studies indicate an interaction between EGR-1 and the *c-myc* promoter leading to upregulation of the *c-myc* gene. Consistent with these observations, ectopic expression of functionally active c-MYC was sufficient to protect the A375-C6 cells from the growth arresting action of IL-1. Our previous studies (Muthukkumar et al., 1996) have defined RB hypophosphorylation as a key requirement for IL-1-inducible G1 phase cell cycle growth arrest. The findings of the present study indicated that rescue of the cells from IL-1-inducible growth arrest by ectopic c-MYC protein is linked to maintenance of RB in the hyperphosphorylated form. Thus, this study has identified *c-myc* as a downstream target of EGR-1 that counteracts growth arrest by preventing RB hypophosphorylation.

Our previous studies have shown that EGR-1 is induced by IL-1 in A375-C6 cells (Sells et al., 1995). Inhibition of either EGR-1 expression by using an antisense oligomer or EGR-1 function by using a dominant-negative mutant enhances the G1 phase growth arrest response to IL-1 (Sells et al., 1995). Consistent with these observations, ectopic expression of EGR-1 results in abrogation of IL-1-inducible growth arrest. A number of previous studies have provided a circumstantial link between EGR-1 induction and a

mitogenic response in diverse cell types (Gashler and Sukhatme, 1995). Moreover, EGR-1 null female mice are infertile, suggesting that EGR-1 is a positive effector in the reproduction process (Lee et al., 1996). In general, these findings suggest a role for EGR-1 in positive modulation of cell growth.

The mechanism by which EGR-1 abrogates growth arrest and thereby positively modulates growth is dependent on the ability of the protein to function as a transcription factor. Although consensus EGR-1-binding sites had been identified in the promoter regions of several growth-associated genes, the transregulation of these gene promoters by EGR-1 had neither been demonstrated nor shown to have a biological significance. The present study used transfection assays to demonstrate that a novel (TCC)_n motif that is found in the promoter regions of many growth-related genes, can confer EGR-1-responsiveness on the *c-myc* promoter. Thus, a role for EGR-1 can be envisioned in the upregulation of other genes, such as those encoding growth factors, growth factor receptors, or protooncogenes, that contain the EGR-1-responsive (TCC)_n motif.

Previous studies of diverse cell types have shown that modulation of *c-myc* expression can directly alter cell growth (Evan and Littlewood, 1993). The demonstration that EGR-1 can transactivate the *c-myc* promoter and upregulate the expression of *c-myc* at the RNA and protein levels suggests a novel mechanism for cell growth regulation by EGR-1. Moreover, the findings suggest that by upregulating the expression of *c-myc*, EGR-1 may regulate the expression of *c-myc*-responsive genes, and thereby expand the number of potential downstream target genes for enhanced signal transduction. Moreover, because there is an overlap in the phenotypic responses to EGR-1 and c-MYC, we

hypothesize, on the basis of the findings of this study, that the overlapping functions are a consequence of a linear regulatory pathway, in which EGR-1 upregulates the expression of *c-myc*. Future studies may test the validity of this hypothesis.

The A375-C6 cells served as an excellent model system for studying the relevance of *c-myc* expression because in response to IL-1 or serum-starvation these cells show a downregulation of *c-myc* expression. Ectopic overexpression of c-MYC abrogated the growth arrest response to IL-1, suggesting that downregulation of c-MYC is functionally required for growth arrest. These findings are consistent with those reported for another growth-inhibitory cytokine TGF β , which causes c-MYC downregulation as part of a growth arrest response in other tumor cells (Alexandrow et al., 1995; Pietenpol et al., 1990). Most importantly, IL-1 shows pleiotropic effects on cell growth: it inhibits the growth of certain tumor cells but stimulates the growth of other tumor cells (cited in Rangnekar et al., 1992). We and others have shown that in fibroblast cells in which it serves a growth-stimulatory signal, IL-1 induces the expression of *c-myc* (Joshi-Barve et al., 1993; Kessler et al., 1992; Rangnekar et al., 1991). The present findings about the functional requirement of *c-myc* downregulation as part of a growth arrest response to IL-1, suggest that the pleiotropic responses to IL-1 may be dependent upon whether IL-1 induces or down-regulates *c-myc* expression. An analysis of *c-myc* expression in a broad panel of IL-1-responsive cell lines whose growth is positively or negatively regulated by IL-1 will help evaluate this hypothesis.

IL-1-inducible growth arrest of A375-C6 cells is associated with an accumulation of hypophosphorylated RB (Muthukkumar et al., 1995), and ectopic overexpression of an EGR-1 target gene product, c-MYC, prevents growth arrest by maintaining RB in a hyperphosphorylated state. This finding is in agreement with those of other studies that have shown that c-MYC can modulate the phosphorylation status of RB (Galaktionov et al., 1996; Marcu et al., 1992). Because c-MYC can modulate the activity of cdk4 by transcriptionally regulating the expression of *cdc25*, a phosphatase that directly controls the activity of cdk4, and because kinase-active cdk4 is required for maintenance of hyperphosphorylated RB (Galaktionov et al., 1996), this pathway should be further investigated to identify the potential mechanism by which c-MYC prevents RB hypophosphorylation in response to IL-1.

V. Concluding remarks

This study has identified a novel mechanism by which EGR-1 counteracts negative growth signals and thereby acts as a positive modulator of growth. The identification of *c-myc*, a key regulator of positive or negative growth responses, as a functional downstream gene target of EGR-1 suggests an important role for EGR-1 in growth control. Thus, by using c-MYC as a downstream target, EGR-1 may

expand the spectrum of its potential target genes and phenotypic endpoints. Because EGR-1 and c-MYC show overlapping biological functions (such as rescue from growth arrest and the stimulation of proliferation, differentiation, or apoptosis), the findings of this study can be extended to determine the effect of the cross-talk on these processes in diverse experimental models.

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