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The transactivation potential of a c-Myc N-terminal region (residues 92–143) is regulated by growth factor/Ras signaling

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ABSTRACT

The colony stimulating factor-1 receptor (CSF-1R) affects mitogenic growth and gene expression in NIH 3T3 cells through signaling pathways that require the products of the c-*ras* and c-*myc* proto-oncogenes. In this work we tested the hypothesis that there is direct communication between the Ras and Myc pathways. In transient transfection assays Ras increased by 5-fold transcriptional transactivation by chimeric c-Myc–Gal4 proteins. A constitutive active form of the CSF-1R also stimulated this activity and co-expression of a dominant negative *ras* gene ablated receptor stimulation. Deletion analysis of the c-Myc N-terminal region demonstrated that amino acid residues between positions 92 and 143 are the targets for Ras action. Transactivation by chimeric Myc proteins that were stably expressed could be transiently enhanced by either CSF-1 or serum, with peak activity occurring 2 h after mitogen stimulation. The steady-state levels of the chimeric c-Myc transactivators were increased following stimulation with CSF-1 or serum, but this increase in steady-state protein level did not strictly correlate with the increase in transactivation activity. Thus, Ras signaling may directly affect the activity of the c-Myc N-terminal region.

INTRODUCTION

The products of the *ras* genes are 21 kDa proteins that bind guanine nucleotides (1). Localization to the inner surface of the plasma membrane is required for the biological activity of Ras (1). In immortalized cell lines, ligand-activated tyrosine kinase receptors are coupled to p21Ras via interaction with the Src homology-containing proteins, such as Grb-2 and the *ras* guanine exchange factor, Sos (2). Thus, the formation of a signaling complex that promotes the generation of the active GTP form of Ras is necessary for mitogenic growth of cultured cells (2).

The product of the c-*myc* gene has been implicated as a key factor in cell growth and differentiation (3). The Myc protein resides in the nucleus and belongs to the basic helix–loop–helix zipper (bHLH Zip) family of transcription factors (3). Myc binds to DNA in a sequence-specific manner (4,5) and possesses two distinct transactivation domains in its N-terminus (6). The Myc polypeptide forms heterodimers with another bHLH Zip protein, the *max* gene product (7). The Myc–Max dimers can transactivate artificial reporters that contain the Myc recognition sequence CACGTG. Transactivation is antagonized when Max dimerizes with another partner, Mad (8).

The *ras* and *myc* oncogenes collaborate to effect neoplastic transformation of primary mammalian cells (9). The interaction of these two genes provides a model for multi-stage carcinogenesis. The molecular basis for this collaboration has not been elucidated. However, the sequences in Myc required for cooperative transformation with Ras coincide with the regions necessary for DNA binding and transactivation (3,10). Therefore, cooperative transformation of primary animal cells requires the combined activation of a signaling cascade and nuclear transcriptional events.

Recent evidence suggests communication between the Ras and Myc pathways during transformation of immortalized cell lines (11–13). When expression of the receptor for colony stimulating factor-1 (CSF-1R), the product of the c-*fms* proto-oncogene, is enforced in NIH 3T3 cells, CSF-1-dependent mitogenic growth and transformation of the cells is promoted (14). CSF-1R mitogenic signaling can be abrogated by direct mutation of a critical autophosphorylation site, Tyr809, in the receptor (15) or by overexpression of either of two dominant suppressors of the Ras signaling pathway, the catalytic domain of the GTPase activating protein (GAP-C; 16) or the DNA binding domain of c-Ets-2 (13). In all three cases, the stimulation of c-Myc by CSF-1 is also abrogated, while expression of the immediate-early genes c-*fos*, c-*jun*, *jun*B and c-*ets*-2 is unaffected (11–13,16). Constitutive expression of c-Myc in these CSF-1-unresponsive cell lines restores ligand-activated mitogenic growth and transformation (11–13). These results indicate that a CSF-1R/Ras signaling pathway is necessary (although not sufficient) for neoplastic transformation of NIH 3T3 cells and that information flows in this pathway through the receptor Tyr809 to Ras to Ets family transcription factors to c-Myc.

One prediction made by this model is that the c-*myc* promoter contains a Ras-responsive enhancer element and that this element contains Ets factor recognition motifs (17–19). In fact, it has been demonstrated that the c-*myc* proximal promoter contains a

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binding site for c-Ets-1 or c-Ets-2 and that c-Ets-1 can transactivate the c-*myc* promoter in transient transfection assays (20). A critical link of Ets proteins to Ras signaling pathways was not demonstrated. In contrast, studies on c-Jun have demonstrated that the activity of this transcription factor is increased by Ras action primarily via post-translational modification of the protein and that this mechanism is central to the collaboration of Ras and c-Jun in transformation of primary rodent cells (21,22). Our recent work with Ets factors also indicates that post-translational alteration of these factors by a Ras signaling pathway is crucial to their action (23). In the present work, we have attempted to assess whether c-Myc activity was affected post-translationally by Ras signaling pathways.

Utilizing transient transfection assays, activated Ras or a constitutively active form of the CSF-1R can increase transactivation mediated by the c-Myc domain located between amino acid residues 92–143 when this domain is fused to the heterologous DNA binding domains of Gal4 or the glucocorticoid receptor. Cells that stably express the c-Myc(41–143) transactivation domain along with an appropriate reporter have been constructed. In these cells, CSF-1 or serum can increase the activity in a temporally regulated manner, with a peak of activity occurring ~2 h after treatment. The stability of the c-Myc fusion protein produced in these cells is increased by growth factor or serum treatment, although the increase is not sufficient to fully account for the elevated activity of the N-terminal domain.

MATERIALS AND METHODS

Cells and culture conditions

NIH 3T3, NIH 3T3 expressing the human CSF-1R (14) and COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 9.5 mM L-glutamine and 100 μg/ml mezlocillin. Purified recombinant CSF-1 was a gift from Steven C.Clark (Genetics Institute, Cambridge, MA). Cells were starved in DMEM plus 0.1% fetal bovine serum prior to stimulation with CSF-1. Cells were stimulated with fetal bovine serum after starvation in RPMI-1640 plus 4 mM L-glutamine, 50 μM β-mercaptoethanol, 100 μM mezlocillin, 400 μg/ml bovine serum albumin, 5 μg/ml insulin, 10 μg/ml transferrin and 5 ng/ml sodium selenite. Stable cell lines were stimulated after 24 h starvation with either 600 U/ml CSF-1 (1 U = 0.44 fmol) or with 30% fetal bovine serum.

Plasmids

The SV40 promoter driven *Gal4–myc* (GM) expression plasmids pGM(1–262), pGM(1–41), pGM(41–103), pGM(103–143) and pGM(41–143) were a gift from Chi V.Dang (Johns Hopkins University) and have been described (6). The GM expression plasmids containing various c-*myc* deletion mutations were generated by the polymerase chain reaction (PCR) using primers based on c-*myc* sequences as previously described (13). Recombinant PCR (24) was used to create a point mutation at Ser62 within a 41–143 context and an internal deletion of codons 92–111 from a 63–143 context. All constructs were sequenced to confirm codon deletions and the proper reading frame with respect to *Gal4*.

All *GR–myc* (GRm) expression plasmids were created by digestion of the *myc* sequences from corresponding pGM constructs with *Sma*I and *Xba*I. *myc* fragments were used to replace v-*myb* DNA sequences from pGR-MYB (25), likewise digested with *Sma*I and *Xba*I.

The reporter plasmid used to assess GM transactivation was pG5E1bLuc (26,27). The GR reporter plasmid, pSK/SL95 contained eight copies of a glucocorticoid-responsive element (GRE) and a herpes simplex virus thymidine kinase (HSV TK) promoter driving luciferase.

Epitope-tagged versions of GRm(41–143) and GRm(S→A62) were expressed via a CMV promoter. The plasmid pCGN (28) was modified by PCR so that it contained two additional copies of the influenza hemagglutinin tag. The triple epitope region was sequenced to confirm the reading frame. The GR DNA binding domain (25) was subsequently cloned in-frame with the triple epitope tag to create pEP3-GR. The EP3-GRm plasmids were generated by subcloning of *myc* sequences from the pGRm vectors described above. Expression vectors for the CSF-1R (29) *ras*N17 (30) and activated *ras* (31) have been previously described.

Transfections

Transient transfection assays were performed as previously described (31).

NIH 3T3 cells expressing the CSF-1R were co-transfected with GM(41–143), pG5E1bLuc and a hygromycin resistance marker by the calcium phosphate method, and individual drug resistant colonies were cloned and analyzed. Stable cell lines were also generated that expressed the triple epitope-tagged versions of GRm(41–143) and the S→A62 point mutation, the pSK/SL95 reporter and a hygromycin resistance gene. Drug-resistant colonies were pooled and the pooled clones used for further analyzes. These cell lines are designated CMV-WT and CMV-62, respectively.

Analysis of RNA and protein expression

RNA was isolated from the WT-10 cultures upon serum stimulation and was subjected to Northern blot analysis as previously described (16). Specific hybridized bands for both probes were quantitated with the Betascope 603 blot analyzer (Betagen, Waltham, MA).

To analyze the expression of different fusion proteins in transient transfections, COS cells were transfected with 10 μg of each pGM plus 1 μg either pHomer6 or pHO6T1 using DEAE–Dextran as previously described (32). Two days post-transfection, cells were metabolically labeled for 4 h with 100 μCi/ml [^{35}S]methionine (Amersham). Cells were lysed in Ab buffer as described (33) in the presence of 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 7.5 μg/ml pepstatin and 10 μg/ml aprotinin. Immune complexes were formed using a polyclonal rabbit serum against the Gal4 DNA binding domain and precipitated with protein A–Sepharose CL-4B (Pharmacia). Immunoprecipitates were fractionated on polyacrylamide gels in the presence of SDS. Gels were treated with Amplify (Amersham), dried and exposed to X-ray film.

For Western blot analysis, treated monolayers were scraped in SDS sample buffer and fractionated on polyacrylamide gels in the presence of SDS. Gels were electroblotted to nitrocellulose and probed with the mouse monoclonal antibody raised against the influenza hemagglutinin epitope (12CA5F; Babco, Berkeley, CA) and detected using the enhanced chemiluminescence method (ECL; Amersham).

RESULTS

Ras stimulates the transactivation potential of an N-terminal domain of c-Myc

Dominant suppression of a mitogenic CSF-1R/Ras signaling pathway abrogates the induction of c-*myc* expression and overexpression of exogenous c-*myc* rescues the mitogenic action of this signaling pathway (11–13). Thus, c-Myc functions as a critical nuclear end point for mitogenic signaling from CSF-1R/Ras. In the case of Ras/c-Jun collaboration the N-terminal activation domain of c-Jun is the critical target for Ras action (21,22). The N-terminus of c-Myc also encodes transactivation domains (6). These domains can be assayed as fusion proteins when joined in-frame to a yeast Gal4 DNA binding domain (6). To assess whether the transactivation domains of c-Myc are also a target for Ras signaling, expression plasmids for Gal4–Myc fusion proteins were analyzed in transient transfections in NIH 3T3 cells in the absence and presence of an activated Ha-c-*ras* allele.

Human c-*myc* coding sequences were expressed in-frame with a yeast Gal4 DNA binding domain under the control of an SV40 early promoter and transactivation by Myc–Gal4 proteins were measured using a luciferase reporter plasmid that contained five copies of the Gal4 DNA binding consensus (see Materials and Methods). Figure 1A depicts portions of the c-Myc transactivation domain that were fused to Gal4 and their relative transactivation potentials in the absence and presence of an activated *ras* allele, while Figure 1B displays a graphic representation of the data. In the absence of a co-transfected *ras* allele, the relative transactivation potentials of the various Myc fusion proteins were comparable with their activities in CHO cells (6). However, co-transfection of an activated *ras* enhanced transactivation by GM(1–262) or by GM(41–143) ~4- to 5-fold in this set of transfection experiments. Transactivation by GM(1–41) was 2-fold, which was the same level as background transactivation of the reporter by Ras in the absence of any exogenous transactivator (Fig. 2B).

Ras did not increase the activity of an SV40-SEAP plasmid (data not shown). In addition, response to the co-transfected *ras* allele was not a function of the basal transactivation potential of the different GM fusion proteins. GM(1–41) was a more efficient transactivator than GM(1–262), but only GM(1–262) responded to Ras (Fig. 1). Thus, the Ras-responsive activity of the Myc transactivation domain is specific to a region encompassing amino acid residues between 41 and 143 of c-Myc.

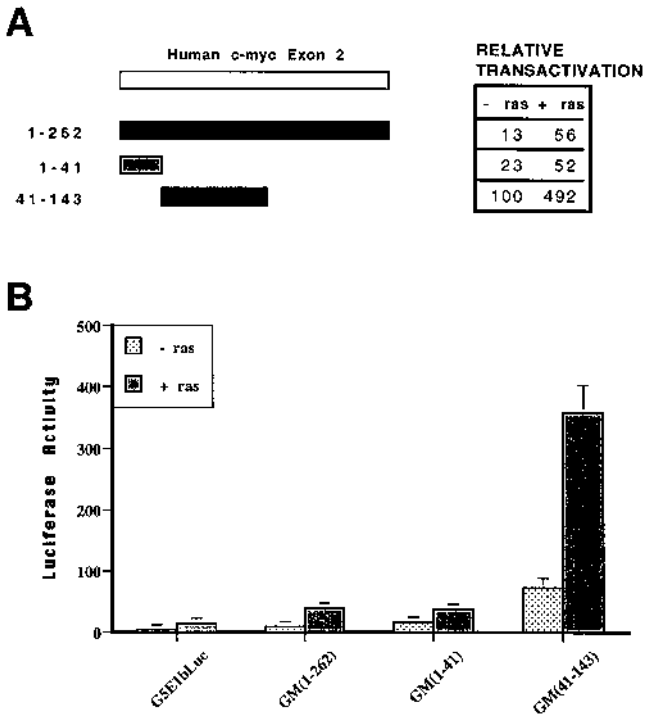

Figure 1. Ras augmented the activity of the 41–143 transactivation domain of c-Myc. (**A**) Shown are the coding sequences of human c-*myc* exon 2 that were assayed in NIH 3T3 cells in transient transfection assays as GM chimeras to delineate sequences that exhibit Ras-enhanced transactivation. Transactivation by the different c-Myc domains is expressed relative to the basal activity of GM(41–143), defined as 100%. Filled boxes depict sequences that were responsive to Ras and stippled sequences were not responsive to Ras. (**B**) Graphic representation of the luciferase data used in the calculation of relative transactivation activities. The data for each construct represent the average of at least three independent transfections where the luciferase activity is corrected for an RSV-SEAP internal control. Error bar indicate the SEM of the experiments.

Mapping the minimal Ras-responsive region in the N-terminal portion of c-Myc

The region between 41 and 143 in the N-terminus of c-Myc was mutagenized in an attempt to further delimit the sequences that conferred Ras responsiveness in the transient assay system. The response of Myc transactivation potential to Ras was lost when the 41–143 region was split into two regions from codons 41 to 103 and 103 to 143 (data not shown for the Gal4 constructs, however see Table 1), suggesting that the target of Ras action might span the region contiguous with amino acid 103. However, because Ser62 of human c-Myc has previously been shown to be critical for transactivation of a c-Myc domain including amino acid residues 1–103 (34,35), we initially focused our attention on this region.

Deletion of amino acids between residues 41 and 62 slightly increased basal activity of the transactivation domain but Ras stimulation of this basal activity was not significantly affected (Fig. 2). A mutation that resulted in the substitution of Ala for Ser at position 62 also had no significant effect on either basal or Ras-induced activities as measured by the transient transfection assays (Fig. 2). In these experiments, the effect of Ras on the Myc 1–41 domain or Gal4 DNA binding domain alone was <2-fold. These data indicate that Ser62 is not the major target of Ras action under our experimental conditions. In contrast, deletion of amino acids 92–111 resulted in both an 80% decrease in basal level activity, as well as a complete loss of Ras-responsiveness (Fig. 2), indicating that this region comprises at least part of the Ras target of the c-Myc N-terminal region.

The c-Myc N-terminal mutations were positioned adjacent to a second heterologous DNA binding domain to validate the results of the transient transfection experiments using GM expression plasmids. The different *myc* coding sequences were subcloned from the GM plasmids to a plasmid encoding a rat glucocorticoid receptor (GR) DNA binding domain (25). These GR–Myc fusion proteins were compared in transient assays (Table 1). Deletion of

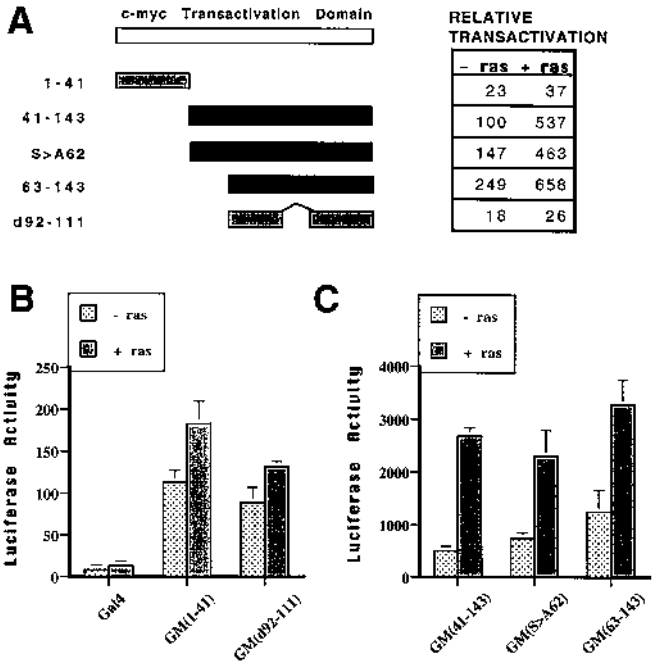

Figure 2. Mapping the sequences of c-Myc required for Ras-enhanced transactivation. (**A**) The structure of mutations in c-Myc domain 41–143 assayed by transient transfection in NIH 3T3 cells as GM chimeras and their relative transactivation potentials in the presence and absence of activated Ras. The activities are again presented relative to the activity of 41–143, which is set as 100%. As in Figure 1, filled regions exhibited Ras-enhanced transactivation, whereas stippled regions were not Ras-responsive. (**B** and **C**) Graphic representation of the luciferase data used in the calculation of relative transactivation activities. GM fusion proteins that exhibited lower activity are presented in (B) and those with higher activity in (C). The data represent the average of three independent experiments where luciferase activity is corrected for an RSV-SEAP internal control. Error bars indicate the SEM of the experiments.

amino acids 41–62 or conversion of Ser62 to Ala62 had no significant effect on Ras stimulation of transactivation potential. Two additional N-terminal mutations were constructed and tested in the GR background. Deletion of amino acids 41–82 gave a phenotype similar to deletion of amino acids 41–62. Truncation of amino acid residues 124–143 severely impaired Ras responsiveness of the c-Myc transactivation potential (Table 1). These data indicate that the region between amino acids 92 and 143 comprises the minimal region of c-Myc that confers responsiveness to the Ras signaling pathway.

Immunoprecipitation using a polyclonal antiserum to the Gal4 domain revealed no appreciable differences in levels of fusion proteins expressed in transiently transfected COS cells (Fig. 3). We chose to use COS cells for this experiment because it is generally accepted that this system provides a reliable way to determine whether mutations affect protein folding (see for example 32). Thus, the various mutations do not result in the production of c-Myc proteins with grossly different stabilities. The difference in apparent mobility between GM(41–143) and GM(S→A62) is an artifact of the cloning of the point mutant pGM(S→A62) arising from fewer codons in the polylinker of this GM chimera. Co-transfection of an activated *ras* allele did not increase the stability of the precipitated proteins (Fig. 3). However, since the cells are growing asynchronously in these transient assays, we could not rule out the possibility that Ras increased protein stability during a specific segment of the cell cycle (see below).

Table 1. Relative transactivation by GR–Myc proteins

Chimeric protein[a]	Basal reporter activity[b]	Reporter activity + Ras[b]	Relative induction[c]
GRm(1-41)	22	35	1.6
GRm(41–103)	15	22	1.5
GRm(103–143)	22	30	1.4
GRm(41–143)	100	642	6.4
GRm(41–143,S→A62)	62	272	4.4
GRm(63–143)	68	267	3.9
GRm(41–143, Δ92–111)	25	38	1.52
GRm(63–123)	20	32	1.6
GRm(82–143)	98	353	3.6

[a]Numbers in parentheses indicate the portion of c-Myc expressed; S→A6, point mutation of residue Ser62 to alanine; Δ92–11, in-frame deletion of amino acids 92–111 in the 41–143 c-*myc* background. See Materials and Methods for details.
[b]Relative luciferase activity with the GRm(41–143) basal activity set as 100. Three independent experiments were performed and the mean of these experiments was used to calculate the values presented.
[c]The activity in the presence of Ras divided by the basal activity.

v-Fms can increase Myc transactivation activity via a Ras pathway

Constitutive, exogenous Ras signaling augmented c-Myc transactivation potential. In order to determine whether activation of an endogenous Ras might likewise affect Myc activity, v-Fms, a constitutively active CSF-1R (29), was used to activate endogenous Ras signaling. Co-transfection of pGRm(41–143) with activated *ras* or v-*fms* resulted in a 6-fold enhancement of transactivation potential (Fig. 4). However, co-transfection of a dominant-negative *ras*N17 (30) resulted in the abrogation of Myc transactivation in response to v-Fms (Fig. 4). Since RasN17 is inhibitory only to normal Ras function (30), the dominant-negative allele did not interfere with enhanced transactivation by activated Ras (data not shown). The response of both Ser62 mutants to v-Fms were similar to those in co-transfections with *ras* (data not shown). Thus, c-Myc transactivation potential is regulated similarly by oncogenic and normal Ras.

Conditional activation of Myc transactivation potential in cells stably expressing the 41–143 N-terminal domain

Stably transfected cell lines were established to evaluate the kinetics of CSF-1R/Ras modulation of c-Myc N-terminal transactivation potential. NIH 3T3 cells stably expressing a wild-type human CSF-1R (14) were co-transfected with the pGM(41–143) expression plasmid, the pG5E1bLuc reporter plasmid and an expression plasmid for a hygromycin B resistance gene (29). Two independent clones, WT-6 and WT-10, were analyzed for the response of Myc transactivation to stimulation with CSF-1 ligand. Quiescent, serum-starved cultures were stimulated with CSF-1 and luciferase reporter activity was measured at various times following growth factor stimulation. CSF-1 stimulation enhanced transactivation by GM(41–143), with reporter activity peaking 2 h post-stimulation (Fig. 5A). The induced level of Myc

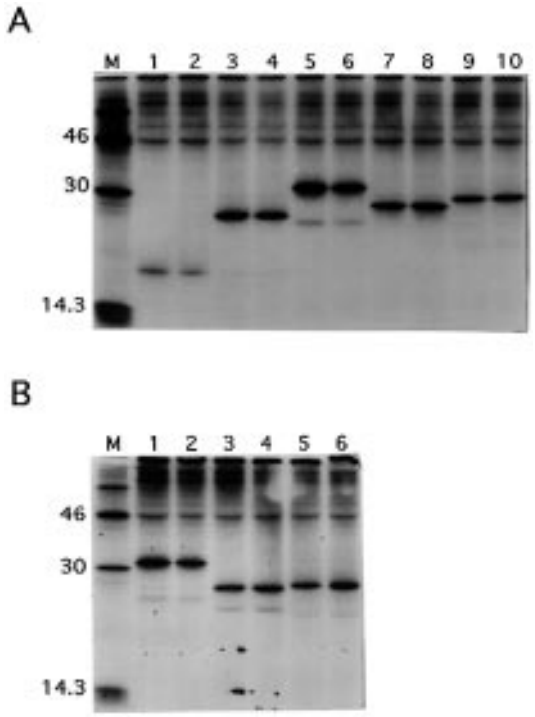

Figure 3. Expression of Gal4–Myc fusion proteins in transient transfections. COS cells were transfected with different *Gal4–myc* expression vectors in the presence of an expression vector for activated Ha-c-*ras* or with the empty expression vector. Cells were metabolically labeled with [^{35}S]methionine and GM chimeric proteins were immunoprecipitated from cell lysates with a polyclonal antiserum against the Gal4 DNA binding domain. Immunoprecipitates were fractionated on SDS-containing polyacrylamide gels. Fluorographs of dried gels are shown. Lanes M in both panels are ^{14}C-methylated protein molecular weight standards, with the sizes of markers (in kDa) indicated to the left of the panels. (**A**) Cells were transfected as follows: Gal4 DNA binding domain (lanes 1 and 2), GM(1–41) (lanes 3 and 4), GM(41–143) (lanes 5 and 6), GM(63–143) (lanes 7 and 8) and GM(S→A62) (lanes 9 and 10). Extracts were prepared from cells co-transfected with an empty expression vector (odd numbered lanes) or with an expression vector for an activated *ras* allele (even numbered lanes). (**B**) Cells were transfected as follows: GM(41–143) (lanes 1 and 2), GM(82–143) (lanes 3 and 4) and GM(d92–111) (lanes 5 and 6). Extracts were prepared from cells co-transfected with an empty expression vector (odd numbered lanes 1, 3 and 5) or with an expression vector for an activated *ras* allele (even numbered lanes).

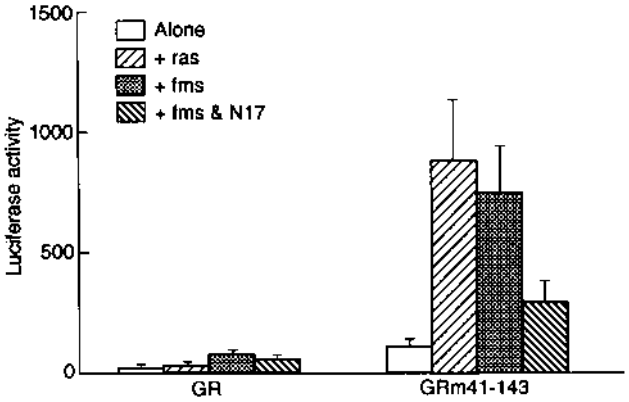

Figure 4. The transactivation potential of c-Myc is enhanced by normal cellular Ras in transient transfections. Transient transfections were performed in NIH 3T3 cells with the pSK/SL95 reporter construct and either pGR (GR DNA binding domain alone) or pGRm(41–143). These constructs were transfected alone, with an activated *ras*, a constitutively active CSF-1R (v-*fms*) or v-*fms* plus the dominant negative *ras*N17. The results of three independent experiments are depicted where the luciferase activity is corrected for an RSV-SEAP internal control. Error bars indicate the SEM of the experiments.

transactivation returned to basal activity within 4–8 h following growth factor stimulation, indicating a tight regulation of Myc transactivation *in vivo* (Fig. 5A). Cells that contained the reporter but not the GM(41–143) expression vector, or an expression vector for only the Gal4 DNA binding domain, showed no response to growth factor treatment. CSF-1 induces peak expression of endogenous c-*myc* mRNA at 1–2 h (12), suggesting that CSF-1 stimulation of quiescent cells leads to coordinate transient increases in c-Myc expression and transactivation potential early in G1.

The response of Myc transactivation was also examined in response to stimulation by fetal calf serum. Quiescent, serum-starved WT-10 cells were stimulated with serum and reporter activity was measure at various times following stimulation. Serum stimulation enhanced Myc transactivation potential to the same extent and with similar kinetics as CSF-1 stimulation (Fig. 5B). Thus, Myc transactivation potential is regulated with similar kinetics by CSF-1 and serum. Furthermore, serum stimulation increased Myc activity when WT-10 cells were infected with a *ras* retrovirus (Fig. 5B). The *ras*-transformed WT-10 cells had a higher basal level of Myc transactivation, however, while the stimulation by serum was reduced compared with cells not harboring an activated *ras* gene, the peak activity of the Myc chimeric transactivator remained tightly regulated and not constitutively elevated. Thus, the Ras pathway appears necessary but not sufficient for regulating the activity of the Myc chimeric protein.

Northern blot analysis of RNA from serum-stimulated WT-10 cells indicated that expression of the *Gal4–myc* RNA was not affected by serum growth factors (Fig. 5C). Similar data were obtained when RNA from CSF-1-treated WT-10 cells was analyzed by Northern blotting (data not shown). Thus, growth factor signaling does not induce expression of the GM(41–143) chimeric gene. Using antisera to the Gal4 domain, expression of the GM(41–143) fusion protein was not clearly detected by immunoprecipitation of proteins from metabolically labeled WT-10 cells or by Western blotting of WT-10 protein extracts (data not shown). The c-Myc protein is characteristically unstable and has a half-life of the order of 30 min (36). Our inability to detect the Myc–Gal4 protein in these cells was due to a high background obtained with the antiserum employed and probably to an inherent instability of the c-Myc peptide produced.

Okadaic acid treatment perturbs the response of Myc transactivation to growth factor stimulation

Myc transactivation potential was tightly regulated in response to either CSF-1 or serum (Fig. 5). If phosphorylation was involved in increasing the activity of the chimeric Myc proteins, then dephosphorylation of proteins might account for the deactivation observed after 2 h stimulation. Thus, inhibitors of serine/threonine protein phosphatase might inhibit the observed down-regulation that occurred. The tumor promoter okadaic acid is a potent inhibitor of the serine/threonine-specific protein phosphatases 1 and 2A (37) and the affect of this compound on the activity of the chimeric Myc protein was analyzed.

Quiescent WT-10 cells were serum stimulated in the presence of increasing concentrations of okadaic acid. Serum alone induced

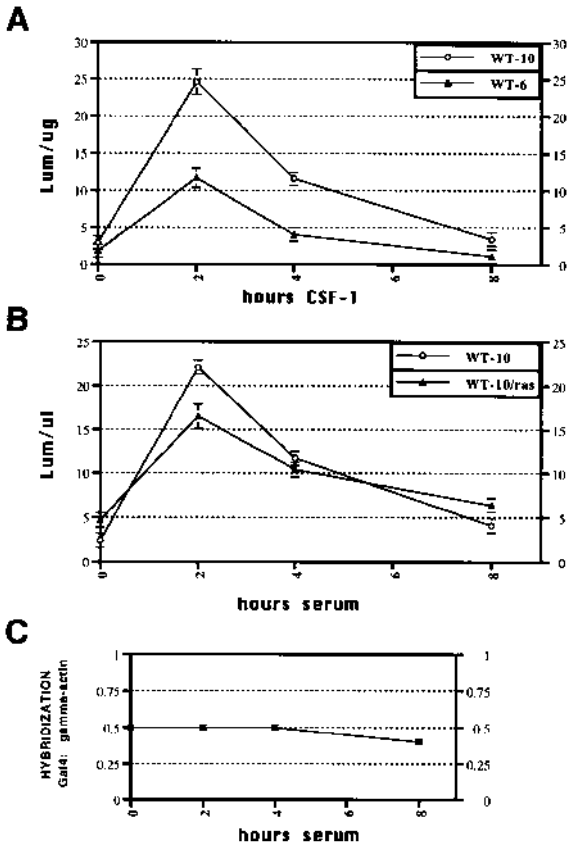

Figure 5. The transactivation potential of c-Myc is enhanced by normal cellular Ras in stably transfected cell lines. CSF-1R-expressing NIH 3T3 cells were stably transfected with pGM(41–143) and the pG5E1bLuc reporter construct and single cell clones were isolated. Cells were serum starved and stimulated with either CSF-1 or serum to activate Ras signaling. (**A**) Serum-starved cells were stimulated with CSF-1 over an 8 h period and extracts were analyzed for luciferase activity at the indicated times. Shown are the results of three independent experiments on two independent clones, WT-6 and WT-10. Error bars indicate SEM. (**B**) Serum-starved WT-10 and *ras*-infected WT-10 cells were stimulated with 30% fetal bovine serum over an 8 h period and extracts were analyzed for luciferase activity at the indicated times. The data shown are the average of a single experiment performed in triplicate. Error bars indicate SEM. Similar results were obtained in multiple experiments. (**C**) Quantitation of a Northern blot of RNA from serum-stimulated WT-10 cells where the blot was probed with Gal4(1–147) and mouse γ-actin radiolabeled DNAs. c.p.m. of Gal4 hybridized bands were standardized to c.p.m. of γ-actin hybridized bands based on quantitation using the Betagen Betascope 603 blot analyzer.

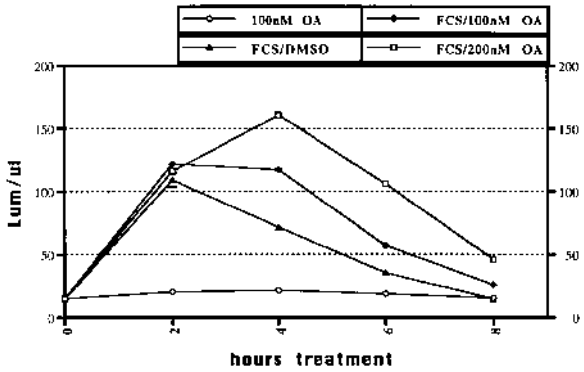

Figure 6. Okadaic acid deregulated c-Myc transactivation potential in stably transfected cells. Serum-starved WT-10 cells were treated with the indicated concentration of okadaic acid and/or 30% fetal bovine serum over an 8 h period. Extracts from duplicate dishes were prepared and analyzed for luciferase activity at the indicated times and the averages are shown. Similar results were obtained in three independent experiments.

Myc transactivation potential with typical kinetics (Fig. 6). In contrast, 100 nM okadaic acid increased the time window of Myc transactivation induced by serum (Fig. 6). Both the time window and the magnitude of the Myc N-terminal activity were potentiated by concomitant treatment of the WT-10 cells with 200 nM okadaic acid and serum (Fig. 6). Okadaic acid treatment in the absence of serum did not affect the N-terminal c-Myc activity. Inhibition of serine/threonine-specific phosphatase disrupts the tight regulation of growth factor-induced c-Myc transactivation potential.

The stability of c-Myc fusion proteins is increased by growth factor/Ras signaling

In order to overcome the technical problems associated with assaying c-Myc N-terminal fusion protein levels in the WT-10 and WT-6 clones (see above), we constructed additional cell lines that expressed the GR–Myc fusion proteins from the potent CMV immediate early promoter. Additionally, this vector allowed the GR–Myc fusions to be epitope tagged with an influenza hemagglutinin peptide antigen (28). Antibody against this antigen produces a lower background in the region the fusion proteins migrate. Pooled clones of cells that expressed both the 41–143 c-Myc domain as well as the 41–143(A62) mutated domain were prepared and assayed.

When stimulated with serum, both CMV-WT and CMV-62 cell lines displayed kinetics for c-Myc N-terminal transactivation identical to those observed in the WT-10 and WT-6 cell lines (Fig. 7A). Similar data were observed upon stimulation of these cell lines with CSF-1 (data not shown). Cells that contained only the luciferase reporter did not respond to serum stimulation (data not shown). No increase in RNA levels of the chimeric genes in response to serum stimulation was observed (data not shown). These data confirm that Ras signaling induces the activity of Myc–Gal4 chimeric products as an early response to serum or CSF-1.

The expression of the epitope-tagged GR–Myc fusion proteins was assessed by Western blot analysis of proteins from CSF-1 treated CMV-WT cells (Fig. 7B). CSF-1 signaling resulted in a transient stabilization of the GR–Myc fusion protein that was partially coincident with increased activity of the c-Myc transactivation domain. The stabilization of chimeric protein was evident at 4 and 8 h after growth factor treatment, when transactivation by this fusion protein had declined (Fig. 7A versus B). A similar protein stabilization was seen upon stimulation of CMV-62 cells (data not shown). Both GR–Myc chimeric proteins were also stabilized by serum stimulation (data not shown). Unlike the data from our transient transfections and immunoprecipitations, where cells were growing asynchronously (Fig. 3), these data indicate that a CSF-1/Ras signaling pathway induces a transient stabilization of the Myc transactivation domain restricted to an early time window after growth factor stimulation. However, the apparent 2- to 3-fold increased level of expressed fusion protein is insufficient to fully account for the 7-fold level of induced transactivation (Fig. 7). In addition, the GR–Myc fusion products remain elevated at 4–8 h post-stimulation, when transactivation activity is largely extinguished. The lack of a tight correlation between chimeric protein levels and transactivation

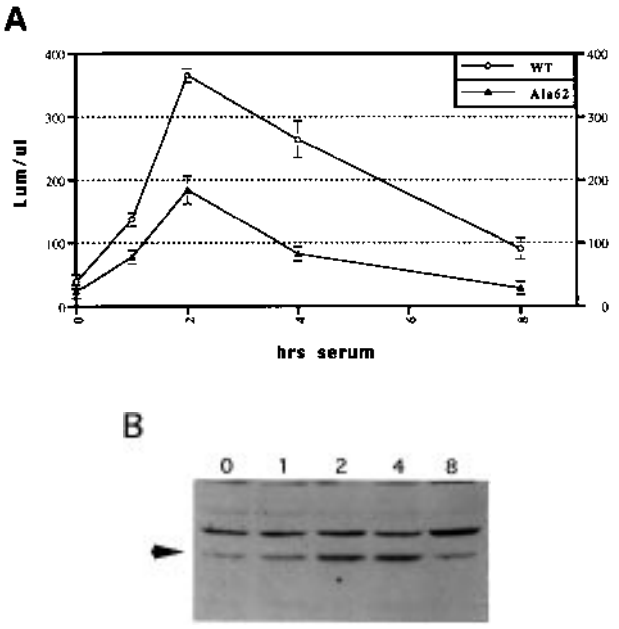

Figure 7. CSF-1/Ras signaling stabilizes the Myc transactivation domain. (**A**) Stable mixed clones of cells containing epitope-tagged versions of GRm(41–143) or GRm(S→A62), as well as the GR-specific luciferase reporter, were analyzed. Cells were serum starved then stimulated with 30% serum and Myc transactivation determined at the indicated times following stimulation. A representative experiment performed in triplicate is shown. SEM of the determinations is indicated by the error bars. (**B**) A Western blot of the antibody-tagged GRm(41–143) from extracts of CMV-WT cells stimulated over time with 250 U/ml CSF-1. Numbers above the lanes indicate the duration of CSF-1 treatment in hours. The band corresponding to the GR–Myc chimera is indicated by the arrow to the left of the blot. The other prominent band is a non-specific, cross-reacting protein (data not shown).

potential indicate that protein stabilization cannot alone account for Ras activation.

DISCUSSION

Collaboration between Ras and Myc suggests that alterations in a minimum of two independent pathways are obligatory events during neoplastic transformation (9). The results presented here indicate that communication at the molecular level between these pathways must also be considered in models describing multi-stage tumor progression. Such communication between a signaling pathway and nuclear transcription factor has previously been revealed in the cooperativity between Ras and c-Jun (21) or Ets-2 (23). At the molecular level, Ras signaling results in phosphorylation at Ser63 and Ser73 in the N-terminal domain of c-Jun (21,22) or Thr72 in Ets-2 (23). Phosphorylation at these sites augments transcriptional transactivation by c-Jun or Ets-2, leading to up-regulation of genes that promote tumorigenicity.

Similarly, in transient assays an active Ras signaling pathway potentiated transactivation by c-Myc chimeric proteins. Amino acid residues 92–143 were defined as a minimal target for Ras action. For co-operative transformation with Ras, amino acids 106–143 of Myc are critical (10). Thus, the Ras-activated region revealed in our experiments correlates well with the region of c-Myc necessary for collaboration with activated Ras (10).

A unique aspect of this work was that the growth factor CSF-1 or serum could stimulate transactivation by this region of c-Myc in a tightly regulated manner. The peak of Myc transactivation activity is coordinate with maximal expression of the endogenous c-*myc* gene product (12). These data suggest that CSF-1R/Ras signaling coordinately regulates the expression and activity of c-Myc upon growth factor stimulation of cells and is consistent with a role of Myc as a competence factor in the early G1 phase of the cell cycle (38). One caveat to this interpretation is that our experiments take advantage of chimeric Myc proteins and artificial gene reporter systems. Studies on authentic early G1 targets for c-Myc, targets yet to be identified, will be required to determine the full biological relevance of our findings.

Previous experiments indicated that overexpression of c-*myc* could rescue deficiencies in the Ras pathway caused by mutation of CSF-1R or by overexpression of GAP-C (11,12), indicating that expression of c-*myc* alone is the critical factor. However, these results are not inconsistent with our present observations in that while Ras signaling was diminished in the published experiments, it was not ablated. In addition, our work indicates that while necessary, Ras signaling alone is not sufficient to fully activate the Myc 92–143 domain. Thus, a residual amount of Ras signaling in combination with additional CSF-1R signaling pathways and overexpression of c-*myc* may be required for the rescue of CSF-1-dependent mitogenic growth in these other experiments (11,12).

Previous reports have indicated that Ser62 of c-Myc can be phosphorylated *in vitro* by MAP kinases and that this residue is involved in the activation of c-Myc transactivation potential in response to EGF signaling (34,39). The requirement for phosphorylation at Ser62 correlates with c-Myc transactivation activity during the G2/M transition in the cell cycle (40), consistent with a requirement for c-Myc in G2/M after EGF stimulation (40,41). However, these data were obtained with amino acid residues 1–103 of c-Myc. For this domain, substitution of alanine for serine at position 62 results in both decreased basal and induced Myc transactivation (34,40). In contrast, deletion through Ser62 or substitution of an alanine at Ser62 only marginally reduced basal transactivation potential in the Myc(41–143) context and these mutations did not abolish the Ras responsiveness of this region. In addition, an increase in c-Myc transactivation activity beyond the early G1 phase of the cell cycle was not observed in our experiments. Because of the different domains assessed in the two sets of experiments, as well as differences in experimental conditions, it may be that two separate activities of c-Myc were measured. In our case, we detect a property of c-Myc manifested in the G1 portion of the cell cycle, while the other experiments with the 1–103 domain measured an activity at the G2/M transition of the cell cycle (40).

Homozygous point mutations in the c-*myc* coding region have been detected in Burkitt's lymphoma (42). Two hot spots for mutation in the N-terminal portion of c-Myc have been identified. Many of these are clustered around Ser62 and Thr58 (42) and often result in loss of either of these phosphorylation sites. The other hot spot for mutation is in the region 85–100. Interestingly, mutations to serine or threonine, as well as conservative changes of threonine to serine are found in the latter region. Therefore, mutations in c-*myc* that result in loss of function as well as gain of function might be expressed in these tumor cells. The effect of these mutations on Myc transactivation activity needs to be determined, but these data might indicate distinct roles for

different portions of the N-terminal region of c-Myc during the generation of Burkitt's lymphoma. This view is consistent with the hypothesis expressed above, i.e. that the regions around Ser62 and 92–111 are necessary for c-Myc activities expressed at distinct portions of the cell cycle.

Phosphorylation of c-Myc may play a role in the cooperativity with Ras, since the phosphatase inhibitor okadaic acid deregulates the growth factor/serum control of Myc transactivation potential. Additionally, the results indicate that the stability of the c-Myc domain is increased by growth factor or serum. The region 83–126 of c-Myc contains an example of a proline/glutamate/serine/threonine-rich sequence (PEST sequence; 43), a sequence involved in determining rapid protein turnover, a characteristic property of proto-oncogene products such as c-Myc. However, the lack of a strict correlation between apparent fusion protein stability and transactivation evident at 4–8 h after CSF-1 stimulation indicate that increased protein stabilization alone cannot account for regulation of Myc activity

An alternative interpretation of these data is that rapid turnover of c-Myc mediated by the PEST sequences is the default and that CSF-1R/Ras action promotes complex formation between c-Myc and other cellular proteins, thereby blocking access of PEST sequences to the default protein degradation machinery. c-Myc has been shown to interact with a number of cellular proteins *in vitro* or *in vivo*, including the Rb-related protein p107 (44) and transcription factors TATA binding protein and TFII-I (45,46) among others. Thus, direct phosphorylation of either c-Myc or of these other factors in response to growth factor/Ras signaling may result in increased complex formation and thus lead indirectly to increased c-Myc stability.

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