A Phosphorylation Site Located in the NH2-terminal Domain of c-Myc Increases Transactivation of Gene Expression*

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Alpna Seth†, Elvira Alvarez‡, Shashi Gupta‡, and Roger J. Davis†‡
From the †Howard Hughes Medical Institute and the ‡Program in Molecular Medicine and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

The c-myc gene encodes a sequence-specific DNA-binding protein (c-Myc) that forms leucine zipper complexes and can act as a transcription factor. Growth factor stimulation of cells causes the phosphorylation of the c-Myc transcriptional activation domain at Ser105 within a proline-rich region that is highly conserved among members of the Myc family (Alvarez, E., Northwood, I.C., Gonzalez, F. A., Latour, D. A., Seth, A., Abate, C., Curran, T., and Davis, R. J. (1991) J. Biol. Chem. 266, 15277–15285). This phosphorylation site is a substrate for growth factor-regulated MAP kinases and for the cell cycle-dependent protein kinase p34<sup>cdk2</sup>. We report that serum treatment of cells results in a marked increase in the transactivation of gene expression mediated by the c-Myc transcriptional activation domain. A point mutation at the site of growth factor-stimulated phosphorylation (Ser<sup>105</sup>) decreases the serum induction of transactivation. These data indicate that the c-Myc transcriptional activation domain may be a direct target of signal transduction pathways.

It has been established that there is an important functional role for Myc proto-oncogene family proteins during cellular proliferation, differentiation, and neoplasia (1). Recently it has been demonstrated that c-Myc is a sequence-specific DNA-binding protein (2–4) that forms leucine zipper complexes (5, 6) and can act as a transcription factor (7). In growing cells the c-Myc protein is constitutively expressed at a low level during the cell cycle (8, 9). The regulation of the biological activity of c-Myc must therefore occur by a post-translational mechanism. One possible mechanism for regulation of c-Myc is provided by phosphorylation (10). Recently, we have reported that Ser<sup>105</sup> is a site of c-Myc phosphorylation that is markedly stimulated by growth factor treatment of cells (11). This site is a substrate for growth factor-regulated MAP kinases and by the cell cycle-dependent protein kinase p34<sup>cdk2</sup> (11). c-Myc may therefore be a direct target of signal transduction pathways. The purpose of the experiments described in this report was to test the hypothesis that Ser<sup>105</sup> is a regulatory site of c-Myc phosphorylation.

The NH2-terminal domain of c-Myc is required for transformation (12) and has been shown to function as a transcriptional activation domain (7). The growth factor-regulated phosphorylation site, Ser<sup>105</sup>, is located within a proline-rich region of the transcriptional activation domain that is highly conserved among members of the Myc family: c-Myc, L-Myc, N-Myc, and s-Myc (11). The location of Ser<sup>105</sup> within this conserved region suggests that phosphorylation of c-Myc at this site may regulate the function of the transcriptional activation domain. To test this hypothesis, we employed a fusion protein strategy (13) in which the NH2-terminal transcriptional activation region of c-Myc (residues 1–103) was fused to the DNA binding domain and nuclear localization signal of the yeast transcription factor GAL4 (residues 1–147) (7, 11). This experimental approach allows the direct examination of the effects of phosphorylation at Ser<sup>105</sup> on the transcriptional activation function of the c-Myc NH2-terminal domain. We report that phosphorylation at Ser<sup>105</sup> is associated with an increase in the level of transactivation of gene expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—The construction of the plasmids pGAL4/Myc, pGAL4/[Ala<sup>105</sup>]Myc, and pGAL4/[Ala<sup>105</sup>]Myc has been reported previously (11). The plasmids pG5ElbCAT and pRSV-Luc were obtained from Drs. M. R. Green and P. Dobner (University of Massachusetts Medical School), respectively. HeLa cells and CHO-K1 cells were obtained from the American Type Culture Collection. [14C]Chloramphenicol was obtained from Amer sham International PLC.

**Transfection of HeLa Cells**—HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. The cells were transfected using the calcium phosphate co-precipitation technique with 5 μg of the activator plasmid (pGAL4/Myc), 10 μg of the reporter plasmid (pG5ElbCAT), and 7 μg of a control luciferase plasmid (pRSV-Luc). The dose response of the activator plasmid was performed using pUC13 as carrier DNA. The transfections were performed with reagents purchased from 5′-3′ Inc. (Boulder, CO) and used according to the manufacturer's directions. Briefly, 5 × 10<sup>5</sup> cells were plated in 100-mm tissue culture dishes 1 day prior to the transfection. The cells were incubated with the calcium phosphate/DNA precipitate for 4 h and subsequently with medium supplemented with 15% glycerol for 2 min. After washing with Tris-buffered saline, the cells were transferred to complete medium and then harvested after 48 h of incubation at 37 °C.

**Transfection of CHO Cells**—CHO cells were grown in Ham's F12 medium supplemented with 5% fetal calf serum. The cells were co-transfected with pGAL4/Myc, pG5ElbCAT, and pRSV-Luc plasmids (2 μg each) using the DEAE-dextran method (7). 5 × 10<sup>5</sup> cells were plated in 100-mm dishes 1 day before transfection. The cells were washed with Tris-buffered saline and incubated with the DEAE-dextran/DNA mixture for 30 min at 22 °C. The medium was then aspirated, and the cells were incubated for 2 min with medium supplemented with 20% dimethyl sulfoxide. After washing with Tris-buffered saline, the cells were incubated for 5 h at 37 °C with medium containing 0.1 mM chloroquine and then washed and transferred to complete medium for 14 h. The cells were trypsinized, seeded into duplicate dishes, and incubated in complete medium for 10 h. The cells were then transferred to serum-free medium and incubated for 18 h. The duplicate dishes of cells were treated without and with 15% DMSO and 0.1 mM chloroquine.

1 The abbreviations used are: CHO, Chinese hamster ovary; CAT, chloramphenicol acetyltransferase.
fetal bovine serum for 2 h prior to harvesting.

Preparation of Cell Extracts—The cells were washed with phosphate-buffered saline (138 mM NaCl, 2.7 mM KCl, 16.5 mM NaHPO₄, pH 7.4) and were harvested by scraping in 3 ml of the same buffer. The cells were collected by centrifugation at 800 rpm for 5 min and the cell pellet was resuspended in 1 ml of extraction buffer (100 mM potassium phosphate, pH 7.8). The centrifugation step was repeated, and the cells were resuspended in 100 µl of extraction buffer. The cells were then lysed by three cycles of freezing on dry ice and thawing at 37 °C. Cell debris was removed by centrifugation in a microcentrifuge for 6 min at 4 °C and the supernatant was stored at −20 °C.

Measurement of CAT Activity—The CAT activity in 25 µl of cell extract was measured using [³⁵S]chloramphenicol, extraction into ethyl acetate, and thin layer chromatography (14). The CAT activity was quantitated using a Phosphorimager (Molecular Dynamics Inc., Sunnyvale, CA).

Measurement of Luciferase Activity—Luciferase activity (15) was measured using 10 µl of cell extract. The sample was mixed with 350 µl of 25 mM glycylglycine (pH 7.8), 15 mM MgSO₄, 1 mM dithiothreitol, and 5 mM ATP in a plastic cuvette. The reaction was initiated by the injection of 100 µl of 1 mM luciferin. Light production was measured for 10 s using a Monolight model 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

RESULTS

The structures of the plasmids used in this study are presented in Fig. 1. GAL4/Myc fusion proteins were constructed (7, 11) by fusing the DNA binding domain and nuclear localization signal of the yeast transcription factor GAL4 (residues 1–147) to the NH₂-terminal transcriptional activation region of c-Myc (residues 1–103). It has previously been demonstrated that this Myc fusion protein is synthesized in tissue culture cells and is correctly localized in the nucleus (7). Transactivation by the GAL4/Myc fusion protein was investigated by employing a reporter plasmid (pG5E1bCAT) that contains GAL4 binding sites adjacent to a minimal adenovirus E1b promoter (TATA) element and the bacterial chloramphenicol acetyltransferase (CAT) gene (14). The activator and reporter plasmids were co-transfected into HeLa cells, and the CAT activity in extracts prepared from these cells was measured. In the absence of the activator (data not shown) or in the presence of GAL4(1–147) no significant level of CAT activity was detected in cell extracts (Fig. 2A). However, a high level of CAT activity was measured in extracts prepared from cells after transient expression of the wild-type GAL4/Myc fusion protein (Fig. 2A). This result confirms the previous observations reported by Kato et al. (7) that the NH₂-terminal region of c-Myc can function as a strong transcriptional activation domain.

To examine the role of the phosphorylation of the c-Myc transcriptional activation domain at Ser²² we used site-directed mutagenesis to replace this residue with Ala (11). Fig. 2A shows that the level of CAT activity observed in experiments using the mutated [Ala²²]Myc fusion protein was reduced compared with the CAT activity measured in experiments using the wild-type fusion protein. In previous studies we have demonstrated that the mutated [Ala²²]Myc fusion protein and the wild-type protein are expressed at a similar level (11). The difference in reporter gene expression (Fig. 2A) is therefore not a result of differences in the level of expression of these c-Myc fusion proteins. This conclusion was confirmed by examination of the dose response of the c-Myc activator plasmids. It was found that the decreased CAT activity caused by the replacement of Ser²² with Ala was observed in experiments employing amounts of the activator
plasmid from 50 ng to 5 μg in the transfection protocol (Fig. 2B).

As a control experiment to examine the possible nonspecific perturbation of protein structure that may be caused by the introduction of point mutations into c-Myc we investigated the effect of the mutation at a different Ser residue (Ser") within the c-Myc transcriptional activation domain. Fig. 2A shows that the replacement of Ser" with Ala caused no significant change in the level of CAT activity. The lack of an effect of the mutation at Ser" in this control experiment suggests that the marked effect of the mutation at Ser" (Fig. 2) is not the result of a nonspecific alteration in c-Myc structure. Instead, these data are consistent with the hypothesis that the defect in phosphorylation at Ser" may account for the reduced level of transactivation caused by the mutated [Ala"/]Myc fusion protein (Fig. 2).

If phosphorylation at Ser" is relevant to transactivation, the increase in Ser" phosphorylation caused by growth factors (11) should alter reporter gene expression in cells transfected with the GAL4/Myc activator plasmid. The effect of the treatment of cells with serum was therefore investigated. It was observed that serum stimulation of HeLa cells (data not shown) and CHO cells (Fig. 3) expressing the wild-type Myc fusion protein caused a marked increase in the level of CAT activity. In contrast, a decrease in the extent of serum-stimulated CAT activity was found in experiments using the mutated [Ala"/]Myc fusion protein in HeLa cells (data not shown) and CHO cells (Fig. 3). A generalized defect in serum-stimulated gene expression does not account for this result because it was observed that serum increased the expression of a heterologous reporter gene (luciferase) that was cotransfected into these cells (Fig. 3). We conclude that the mutation of Ser" causes a specific reduction in serum-stimulated gene expression mediated by the c-Myc transcriptional activation domain. This result is consistent with the hypothesis that the phosphorylation of the c-Myc activation domain at Ser" increases transcription.

**DISCUSSION**

The MAP kinases represent a family of enzymes that are thought to have an important role during signal transduction (16, 17). A signal transduction pathway has been described in which the MAP kinases are activated by phosphorylation at Thr and Tyr (18, 19) by a protein kinase cascade mechanism (20). The MAP kinases can phosphorylate and activate the S6 protein kinase (21), but the physiologically relevant targets of this signal transduction pathway are poorly understood (22). We have previously reported that c-Myc is phosphorylated at Ser" by a MAP kinase in vitro (11). The rapid growth factor-stimulated phosphorylation of Ser" observed in serum-starved cells indicates that c-Myc may be a physiological substrate for the MAP kinases (11). We therefore propose the working hypothesis that the c-Myc transcriptional activation domain may be a direct target of the MAP kinase signal transduction pathway that regulates gene expression (Fig. 4). Furthermore, as c-Myc is phosphorylated at Ser" by the cell cycle-dependent protein kinase p34"/c-c (11), it is also possible that the phosphorylation and transactivation potential of c-Myc is regulated in proliferating cells during the cell cycle.

Recently, substantial evidence has been reported indicating that phosphorylation is an important mechanism for the regulation of transcription factor function. For several transcription factors it has been demonstrated that DNA binding activity is regulated by phosphorylation (e.g. SRF (Ref. 23), c-Myb (Ref. 24), and c-Jun (Ref. 25)). However, phosphorylation can also regulate transcription factor function independently of changes in DNA binding activity. For example, the activity of CREB is stimulated by phosphorylation by the cyclic AMP-dependent protein kinase at a specific site within the activation domain (26). Phosphorylation of the activation domain of c-Jun has also been proposed to increase transcription (27). This mechanism of regulation of CREB (26) and c-Jun (27) by phosphorylation may be similar to the regulation of the c-Myc transcriptional activation domain caused by phosphorylation at Ser" described in this report. The phos-
Phosphorylation of the c-Myc Transactivation Domain

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