c-Myc Does Not Require Max for Transcriptional Activity in PC-12 Cells

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INTRODUCTION

Both nerve growth factor (NGF) and epidermal growth factor (EGF) induce the transient transcription of the proto-oncogenes c-myc, thought to be involved in cellular proliferation and differentiation (1–3). The product of the c-Myc proto-oncogene is a basic helix–loop–helix leucine zipper (b/HLH/LZ) protein (4). This structure is characteristic of DNA binding and protein dimerization domains of transcriptional regulators (5). Myc proteins do not form homodimers and bind DNA inefficiently, if at all, under physiological conditions (6–8), but do bind the DNA sequence CACGTG upon dimerization with a partner, Max, mediated through an interaction with the b/

HLH/LZ regions of both proteins (9–13). Two major forms of Max have been described, both of which can heterodimerize with c-Myc in vivo and in vitro. In the absence of Myc, Max itself can homodimerize and bind with high affinity to the same DNA sequence as the Myc/Max heterodimer (9–13). Only the Myc/Max heterodimer can activate while Max/Max homodimers repress the transcription of a reporter gene containing a Myc/Max DNA binding site (14, 15).

Max is a stable protein and is constitutively expressed, in contrast to Myc which is highly regulated and has a short half life (16, 17). Both Myc and Max are phosphoproteins in vivo (18) and in vitro (19, 20), but the biological significance of this phosphorylation is not yet understood. The obligate interaction between Myc and Max and their differential regulation led us to study the role of these two genes in PC-12 cell differentiation and growth. Here we show that Max mRNA or protein expression is not detected in PC-12 cells. However, NGF-induced c-Myc or overexpressed c-Myc can regulate the transcription of a reporter gene harboring a c-Myc/Max DNA binding site. Functional c-Myc in PC-12 cells therefore may associate with another dimerization partner in order to activate transcription.

MATERIALS AND METHODS

Materials. NGF 2.5S was purchased from Bioproducts for Science (Indianapolis, IN). Anti-Max antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). Mouse monoclonal anti-c-Myc antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Protein G-agarose was from Oncogene Science (Uniondale, NY). [125I]-Labeled protein A (30 mCi/mg), [125I]-labeled anti-mouse IgG (5–20 mCi/mg), [α-32P]dATP (3000 mCi/mmol), and [35S]methionine/cysteine (1113 Ci/mmol) were from Amersham Co. (Arlington Heights, IL). (1,2,4-C)-d-threo- chloramphenicol (61 mCi/mmol), [32P]orthophosphate (285 Ci/mg), and Dulbecco's modified Eagle's medium without phosphate or methionine/cysteine were from ICN

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Radiochemicals (Irvine, CA). All other reagents were purchased from Sigma (St. Louis, MO) and were the highest quality available.

**Cell culture.** PC-12 cells and NIH 3T3 expressing trk (3T3-c-trk) were as described (22, 23). Rat fibroblasts (3Y1) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY) and penicillin and streptomycin (100 μg/ml). Human erythroleukemia cells, K562, were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin, and streptomycin (100 μg/ml).

**Immunoprecipitation and immunoblot analysis.** Cells grown in 150-mm dishes were washed twice with ice-cold PBS and lysed in 1 ml of ice cold RIPA buffer (10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton, 1% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, aprotinin, and leupeptin (10 μg/ml) on ice for 20 min. K562 cells were harvested and washed by centrifugation prior to lysis. Lysates were precleared with 50 μl of Panasorbin (Calbiochem, San Diego, CA) and centrifuged at 10,000g for 10 min. Supernatants were collected and protein concentrations were determined by the Bio-Rad Bradford assay (Bio-Rad Laboratories, Richmond, CA). Equal protein concentration of lysates derived from PC-12 cells, 3T3-c-trk and 3Y1 cells (1.5 mg), and 0.75 mg of lysate protein from K562 cells were incubated with anti-Max antibodies. After 16 h at 4°C, immunoprecipitates were mixed with protein A–agarose for 2 h and the immunocomplexes bound to the beads were washed twice with RIPA buffer, once with 500 mM LiCl, 10 mM Tris–HCl (pH 7.4), and finally with water. The immunocomplexes were solubilized at 100°C for 3 min in 25 μl Laemmli sample buffer. Proteins were resolved on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and immunoblotted with anti-Max antibodies. Bound antibodies were detected with 125I-labeled protein A followed by autoradiography. For c-Myc protein analysis, PC-12 cells were starved for 12 h and then stimulated with serum (10%) for 1 h or NGF (10 nM) for the indicated times. Starved 3Y1 cells were stimulated with serum (10%) for 1 h. The cells were lysed with RIPA buffer and immunoprecipitated with anti-c-Myc monoclonal antibodies. After 12 h at 4°C, immunoprecipitates were collected with protein G–agarose and separated on SDS–PAGE (12%). Immunoblots were developed with 125I-labeled antimouse IgG.

**In vivo labeling of cells.** The metabolic labeling of cells with [35S]methionine/cysteine (100 μCi/ml) was performed in methionine cysteine-free DMEM. After 15 h incubation, cells were lysed with RIPA buffer followed by the anti-Max immunoprecipitations and analyzed by SDS–PAGE (15%). The gels were treated with Amplify (Amersham) to enhance the detection of labeled proteins.

For labeling with [32P]orthophosphate, cells were grown in 35-mm dishes and labeled for 3 h with 500 μCi/ml in phosphate free DMEM at 37°C. Extraction of cells and immunoprecipitations were done as described above.

**Northern (RNA) blot analysis.** PC-12 cells were serum starved for 12 h and treated with serum (10%) for 1 h or NGF (10 nM) for the indicated times. Cytoplasmic RNAs were isolated by the acid quanidinium thiocyanate–phenol–chloroform extraction method (24). RNA samples (15 μg) were electrophoresed in 1% agarose, 2.2 M formaldehyde and transferred to nylon membrane (Gibco-BRL) by capillary diffusion. The transfer membrane was prehybridized for 4 h and hybridized overnight at 42°C with the 300-bp [nucleotides 42 to 315 (9)] Max probe, which was gel purified and labeled with [α-32P]dATP by random priming at a sp act of 1 × 10⁹ dpm/μg. Following hybridization, the blot was washed at room temperature in 2× SSC, 1% SDS (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) and then washed twice in 0.5× SSC, 1% SDS at 65°C 30 min each time. Equal loading of RNA was determined by ethidium bromide staining of rRNA.

**Transient expression assay.** The reporter plasmid was constructed by ligating 45 oligonucleotides containing two putative Myc/Max DNA binding sites (9) and driving the transcription of the chloramphenicol acetyltransferase (CAT) gene from the thymidine kinase promoter (−43 to +45). The sequence is as follows (the DNA binding sites are underlined): 5′-CTAGAGTCGACAGTCAGTGCTTGTCGCTATCGCTAGTCGAGTACGTCAGTTT-3′ (generously provided by Dr. Colin Godin, Marie Curie Research Institute, UK). One day before transfection, PC-12 cells were harvested and plated at 60–70% confluency in 100-mm dishes. The cells were transfected with the reporter gene (10 μg), together with 2 μg of the control plasmid β-galactosidase expression vector pCH110 (Pharmacia LKB) using Transfectam (Promega, Madison, WI). Cells were incubated with the DNA for 4 h in serum-free DMEM. The DNA was removed, the cells were fed with DMEM containing 10% FBS and incubated for 24 h. After additional incubation in serum-free DMEM for 12 h, the cells were stimulated with 10 nM NGF or 10% serum for 4 and 8 h. CAT activity of duplicate cultures was determined as described (25). Reaction mixtures containing 25 μg protein were incubated at 37°C for 4 h. [3H]Chloramphenicol acetylation was quantified by counting directly the TLC plates using a Bioscope 603 blot analyzer (Betagen Co., Waltham, MA). The cell lysates were assayed for β-galactosidase activity in 200 μl reaction mixture containing 25 μg protein using chlorophenol red β-d-galactopyranoside (Boehringer-Mannheim, Indianapolis, IN) as a substrate (26) at 37°C until a faint red color appeared, and the optical density was measured at 595 nm. To control for efficiency of transfection, the normalized CAT activity was calculated by dividing the counts per minute of the acetylated forms...
by the measured OD. For the myc dose–response experiments, each plate of PC-12 cells received 10 μg of the reporter gene and 2 μg of the vector pCH110, along with the indicated amounts of pKOMyc construct in which the transcription of the mouse c-myc gene is driven from the SV40 promoter [generously provided by Dr. Ronald A. DePinho (27)]. The total DNA in all these transfections was maintained at 50 μg using REP10 (Invitrogen, San Diego, CA). In experiments using CMVMyC construct, which is a murine c-myc gene linked to the cytomegalovirus (CMV) promoter [generously provided by Dr. Michael D. Cole (19)], the total amount of DNA was maintained at 50 μg using CMV plasmid lacking c-Myc. At 48 h after transfections, cells were harvested and transcriptional activation was assayed as described above.

Statistical analysis. Results were analyzed by single factor ANOVA.

RESULTS

Because of the proposed central role for the transcription of c-Myc in the cellular actions of NGF and EGF, we evaluated the regulation of c-Myc transcription activity by complex formation with Max in PC-12 cells, a model system for growth and differentiation of neuronal cells. Thus far, Max mRNA and protein have been detected in all tissues in which c-Myc is expressed (17, 18, 28, 29). Moreover, Myc/Max heterodimeric complexes have been found in human neuroblastomas and other leukemia cell lines where Myc is overexpressed (17, 18). To explore the role of Myc/Max complexes in growth factor-induced gene expression, we first evaluated PC-12 cells for Max expression. RNA was isolated from quiescent PC-12 cells stimulated with NGF or serum for various periods of time. As shown in Fig. 1, we observed two RNA species isolated from rat (3Y1) cells, a major RNA species of 2.0 kb, and a minor species of 1.7 kb, that hybridized specifically with a probe from the Max cDNA. These two species of RNA have been previously observed in other murine cell lines (28). However, Max mRNA was not detected in PC-12 cells. Moreover, RNA transcripts were not induced after exposure of these cells to NGF or serum stimulation. Max protein levels were also evaluated (Fig. 2). Lysates prepared from growing PC-12 cells were immunoprecipitated with anti-Max antibodies or non relevant rabbit serum followed by SDS–PAGE and immunoblotting with the same anti-Max antibodies. In control experiments, Max proteins were immunoprecipitated from K562 human erythroleukemia cells, NIH3T3 cells expressing c-trk (3T3-c-trk), and 3Y1 rat fibroblasts. Max was readily detected as a doublet migrating at around 20–22 kDa in K562, 3T3-c-trk, and 3Y1 cells, but could not be immunoprecipitated from PC-12 cells under these conditions (Fig. 2A). Moreover, cells were labeled with [35S]methionine/cysteine (Fig. 2B) or [32P]orthophosphate (Fig. 2C) prior to immunoprecipitation with anti-Max antibodies. As described above for immunoblot analysis, the Max doublet could be detected in K562, 3T3-c-trk, or 3Y1 cells, but not in PC-12 cells.

To further investigate c-Myc function in PC-12 cells, we examined c-Myc induction by NGF. Quiescent PC-12 cells were treated with 10 nM NGF or 10% serum for the indicated times and quiescent 3Y1 cells were treated with 10% serum for 1 h. Lysates were prepared and were im-

FIG. 1. Analysis of Max RNA in PC-12 cells and rat 3Y1 fibroblast. Cytoplasmic RNA (15 μg) was isolated from growth-arrested PC-12 cells (Q) or cells stimulated with serum (10%) for 1 h or with NGF (10 nM) for the indicated times, and from quiescent 3Y1 cells. RNAs were separated and hybridized to 32P-labeled Max-specific probe as described under Materials and Methods. The positions of 28S and 18S rRNA and of Max RNAs (2.0 and 1.7 kb) are shown. M, RNA size markers (in kb).

FIG. 2. Identification of Max protein (p20/21) in cell extracts. (A) Lysates from growing PC-12 cells, 3T3-c-trk, rat 3Y1 fibroblast (1.5 mg), and K562 human erythroleukemia cells (0.75 mg) were precipitated with either anti-Max antibodies or nonrelevant (anti-c-trk) antibodies (NR) as indicated. Proteins were separated on 15% SDS–PAGE before immunoblotting with anti-Max antibodies. PC-12, 3T3-c-trk, and K562 cells were labeled with (B) [35S]methionine/cysteine or (C) [32P]orthophosphate prior to immunoprecipitation with anti-Max or nonrelevant antibodies as indicated. Proteins were separated on parallel 15% SDS–PAGE and subjected to autoradiography. Molecular weight markers (in kDa) are indicated at left.
munoprecipitated with anti-c-Myc antibodies. Immunoprecipitated proteins were subjected to SDS-PAGE followed by immunoblotting with the same antibodies (Fig. 3). Both NGF and serum led to stimulation of c-Myc protein expression within 30 min of exposure in PC-12 cells. In PC-12 cells, the major band exhibited an apparent molecular weight of 55–60 kDa, with a minor band migrating at 35–40 kDa representing a c-Myc degradation product (18). In rat SY1 cells the major c-Myc band exhibited an apparent molecular weight of 65–70 kDa. The intense 50-kDa band represents one of the mouse immunoglobulin heavy chains from the antibodies.

The lack of Max expression in this PC-12 cell line led us to examine the transcriptional activity of c-Myc in these cells. In order to determine whether NGF or serum-dependent c-Myc induction is functional in the absence of Max in PC-12 cells, we measured the transcriptional activity of a CAT reporter construct bearing a Myc/Max-dependent promoter sequence. In quiescent PC-12 cells, moderate levels of reporter gene expression were observed, probably due to low level of basal expression of Myc or other transcription factors, such as the upstream stimulation factor (USF) that binds to the same sequences (30). Treatment of PC-12 cells with NGF or serum resulted in a two- to threefold induction in the target gene expression over quiescent cells (Fig. 4). Moreover, cotransfection of PC-12 cells with the Myc/Max binding site reporter gene construct and vectors that overexpressed c-Myc protein generated a consistent increase in the target gene expression (Fig. 5). No increase in CAT activity was observed when c-Myc was cotransfected with a reporter plasmid lacking the Myc/Max DNA binding site (control in Fig. 5B). The observations presented here implicate that c-Myc functions as a transcription factor and affects gene expression in PC-12 cells in response to differentiative or mitogenic signaling.

**FIG. 3.** Kinetics of c-Myc protein synthesis in PC-12 cells. PC-12 cells were serum-starved for 12 h and then treated with serum (10%) for 1 h or NGF (10 nM) as indicated. Following the treatments, lysates were prepared with RIPA buffer as described under Materials and Methods and immunoprecipitated with anti-c-Myc antibodies. Immunoprecipitated proteins were separated on 12% SDS-PAGE followed by immunoblotting with the same anti-c-Myc antibodies. The position of c-Myc proteins are indicated. The intense 50-kDa band represents a mouse immunoglobulin heavy chain from the antibodies. Molecular weight markers (in kDa) are indicated at left.

**FIG. 4.** c-Myc/Max DNA binding site-dependent transcription in PC-12 cells. PC-12 cells were cotransfected with the reporter CAT plasmid containing two Myc/Max DNA binding sites (10 μg) and β-galactosidase expression vector (pCH110, 2 μg). The cells were serum starved 24 h after transfection and then stimulated with serum (10%) or NGF (10 nM) or left untreated (control). CAT activity (counts per minute of the acetylated forms) was normalized for differences in transfection efficiency measurement by the β-activity (OD value) as described under Materials and Methods, and are presented as counts per minute/OD value. Each data point represents the mean from duplicate samples ± SD significant at *P < 0.00005* by single-factor ANOVA. This experiment was repeated three times with consistent results showing serum- and NGF-dependent activation of c-Myc transcriptional activity.

**DISCUSSION**

Although the nuclear proto-oncogene c-Myc is likely to play a central role in both cellular proliferation and differentiation in response to extracellular stimulation (3, 4, 31, 32), the precise molecular mechanisms involved in these different effects remain poorly understood. c-Myc is one of the early immediate genes that is rapidly and transiently induced upon treatment of PC-12 cells with both the differentiative factor NGF and growth factors such as EGF (1). It has been shown previously that c-Myc overexpression in PC-12 cells failed to induce differentiation, but instead caused cells to proliferate in response to NGF (33). This observation is distinctly different from the findings with overexpression of oncogenic src, ras, and raf which directly induce differentiation of PC-12 cells (34–36). To explore the function of c-Myc in
more detail in PC-12 cells, we examined NGF-induced c-Myc transcription activity by evaluating complex formation of c-Myc with Max. c-Myc has been shown to function as a transcription factor upon dimerization with Max (14, 15). However, no Max mRNA or protein could be detected in PC-12 cells, despite the NGF and serum-responsive expression of c-Myc. The lack of detectable Max expression in this cell line allowed us to evaluate the absolute requirement of this protein in transcriptional activity. Transfection of PC-12 cells with a reporter construct containing a Myc/Max DNA binding sequence demonstrated that Myc protein induced by NGF or serum in these cells was able to mediate the transcriptional activation of a reporter gene (Fig. 4). Furthermore, introduction of c-Myc vectors into PC-12 cells generated an increase in the reporter gene activity over the endogenous levels, indicating that Myc is capable of regulating gene expression in these cells, even in the absence of Max expression. These properties of overexpressed c-Myc are comparable to those observed in cell lines where Max is constitutively expressed (14, 15).

Because c-Myc alone neither forms homo-oligomers nor binds DNA in an efficient manner (6-8), its ability to activate transcription in PC-12 cells lacking Max is presumed to occur with another dimerization partner in these cells. Other Max-like proteins such as Mad and Mxi1 have been identified (37, 38). However, these proteins interact specifically with Max to bind the same Myc/Max DNA binding site. The N-terminal domain of c-Myc has been shown to mediate binding to the product of the retino-blastoma gene pRB (39). Although we have not yet characterized the nature of the Myc heterodimer in PC-12 cells, the possible interaction of Myc with the transcription initiation factor TFII-I has recently been suggested (40), although an in vivo association between Myc and TFII-I remains to be demonstrated. Our data demonstrate that Myc is transcriptionally active in PC-12 cells, possibly involving another yet unidentified protein in transcription regulation by Myc.

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REFERENCES


