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Omomyc, a Potential Myc Dominant Negative, Enhances Myc-induced Apoptosis[1]

Laura Soucek, Richard Jucker, Laura Panacchia, Ruggero Ricordy, Franco Tatò, and Sergio Nasi[2]

Centro Acidi Nucleici Consiglio Nazionale delle Ricerche [L. S., R. J., L. P., S. N.], Centro di Genetica Evoluzionistica Consiglio Nazionale delle Ricerche [R. R.], Dip. Biologia Cellulare e dello Sviluppo [F. T.], Università La Sapienza, P.le A. Moro 5, 00185 Roma, Italy

ABSTRACT

The Myc basic helix-loop-helix zipper domain determines dimerization with Max and binding to the DNA E-box, both of which play a critical role in Myc regulation of growth, proliferation, tumorigenesis, and apoptosis. The mutant basic helix-loop-helix zipper domain, Omomyc, dimerizes with Myc, sequestering it in complexes unable to bind the E-box, and so acting as a potential dominant negative. Consistent with this, Omomyc reverses Myc-induced cytoskeletal disorganization in C2C12 myoblasts. Surprisingly, however, Omomyc strongly potentiates Myc-induced apoptosis in a manner dependent on Myc expression level. Expression analysis of known Myc target genes indicates that Omomyc inhibits transcriptional activation but enhances repression. These findings suggest that Omomyc can selectively trigger apoptosis in cells overexpressing Myc, possibly through the transcriptional repression of specific genes.

INTRODUCTION

The mechanisms by which Myc regulates cell growth, proliferation, apoptosis, and tumorigenesis remain obscure (1–4). Myc is a transcription factor that binds to the E-box DNA consensus sequence CACGTG as a heterodimer with its stable partner protein Max. Myc homodimers are unstable and bind DNA poorly. Myc dimerization with Max and DNA binding are both determined by its evolutionary conserved bHLHZip[3] domain at the protein's COOH terminus. bHLHZip domains are present in, and essential for the function of, all proteins of the Myc/Max/Mad network (5, 6). The NH_2-terminal region of Myc contains a transcriptional activation domain together with several small and highly conserved "Myc boxes" that are present in Myc proteins in all vertebrate species. One of these, Myc box II, together with the bHLHZip have been implicated in the recruitment of chromatin remodeling complexes and other cofactors, although it remains unclear how such interactions modulate Myc/Max target gene expression (1, 4, 7).

Omomyc is a Myc-derived bHLHZip domain obtained by substituting four amino acids in the Myc zipper that were identified as obstacles to Myc homodimerization. The amino acid substitutions E57T, E64I, R70Q, and R71N are responsible for the altered dimerization specificity of Omomyc, which homodimerizes efficiently (8). Furthermore, Omomyc forms heterodimers with wild-type c-Myc, interfering with the formation of Myc/Max dimers and suppressing binding to E-box elements. As a consequence, Omomyc suppresses activation of artificial E-box promoter elements by Myc/Max and inhibits colony formation in NIH3T3 cells, acting as a *de facto* dominant negative of Myc (8). In this study, we have used Omomyc as a probe to interfere with two distinct functions of Myc: (*a*) cytoskeletal reorganization; and (*b*) apoptosis, which can be involved in the development or maintenance of tumors (9).

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[2] To whom requests for reprints should be addressed, at La Sapienza University, Centro Acidi Nucleici Consiglio Nazionale delle Ricerche, P. le A. Moro 5, 00185 Roma, Italy. Phone: (39) 0649912241/27; Fax: (39) 0649912500; E-mail: sergio.nasi@uniroma1.it.

[3] The abbreviations used are: bHLHzip, basic helix-loop-helix zipper; FACS, fluorescence-activated cell sorter; 4-OHT, 4-hydroxytamoxifen; ER, estrogen receptor.

MATERIALS AND METHODS

Expression Vectors and Cell Lines. The Omomyc DNA sequence was PCR amplified from pCSOmomyc (8) and inserted, in frame, into pBS+ER (10). After amplification, the Omomyc-ER, or Omomer, chimeric gene was cloned into the retroviral vector pBabePuro. C2C12, Myc-C2C12 (11), Rat1, and Rat1 *myc*$^{-/-}$ cells (from J. Sedivy) were infected with empty and Omomer or c/vMyc (pBabeNeo-c/vMyc) retroviruses and selected with appropriate antibiotics. 4-OHT (Sigma) was used at 5×10^{-7} M.

Western Blotting. Cells were harvested by centrifugation, washed in PBS, and lysed with SDS gel sample buffer at 85°C. The lysates were subjected to 10% SDS-PAGE and blotted to polyvinylidene difluoride membrane (Amersham). Omomer was stained with anti-ER antibody (Santa Cruz Biotechnology; MC20, 1:1000) and Myc with anti-Myc antibody (1537 from R. Eisenman, 1:1000), followed by protein A-peroxidase (Sigma; 1:5000) and development with enhanced chemiluminescence kit (Amersham).

Immunohistochemistry and FACS Analysis. Cells were washed twice in PBS, fixed in methanol/acetone (1:1) for 10′, and permeabilized with 0.25% Triton X-100. Actin filaments were stained with FITC-phalloidin (Sigma) for 30′; nuclei were stained with 0.1 μg/ml Hoechst 33258 (Sigma). Samples were mounted in PBS/glycerol and observed under a fluorescence microscope. Flow cytometric analysis was carried out with a FACStar cytometer (Becton Dickinson) on cells (10^6/ml) fixed in ethanol and resuspended in PBS containing 20 μg/ml propidium iodide.

Gel Mobility Shift Assay. Cells extracts were prepared in F-buffer (12), 300 μl/10-cm dish. The CV13/14 oligonucleotide 5′-GATCCCCCCAC-*CACGTG*GTGCCTGA was used as probe. Extract (2–5 μl) was incubated for 30′ at 25°C with 0.1–0.5 ng of labeled oligonucleotide and 1 μg of poli-dIdC in 15 μl of GS-buffer (12). Competitor oligonucleotide was added at a 200-fold excess, and an additional 15′ incubation was performed. For supershift analyses, 1 μl of anti-Max (C124; Santa Cruz Biotechnology), anti-Myc (1537, R. Eisenman), or control antiserum was added for 15′.

Northern Blotting. For exponential growth studies, cells were harvested 24 h after plating. For serum stimulation experiments, cells were cultured in 0.1% serum for 48 h, the medium was replaced with 10% serum medium, and cells were harvested at different time points. Total RNA was isolated with Trizol (Life Technologies, Inc.), separated on agarose/formaldehyde gels, and blotted to Nylon membranes (Amersham). Blots were hybridized in 50% formamide at 42°C with radiolabeled cDNA probes. Blots were washed in 2 × SSC, 0.1% SDS at room temperature, and 1 × SSC, 0.5% SDS at 50°C. Quantitation of bands was done with a Molecular Dynamics Instant Imager.

RESULTS

A conditional Omomyc protein was used to understand the functional consequences of interfering with Myc. The conditional protein, Omomer, was derived by fusing the Omomyc coding sequence with the sequence of mutant murine ER (ER or Mer) ligand-binding domain, activatable by 4-OHT (10). The chimeric gene was cloned into the retroviral vector pBabePuro. The effects of Omomyc expression were then analyzed in C2C12 myoblasts, a cell line model for muscular differentiation in which the phenotypic consequences of Myc overexpression have been thoroughly described (11). Normal and Myc-transformed (Myc-C2C12) myoblasts were infected with either control or Omomer retroviruses and selected with puromycin. Neither cell morphology nor proliferation rate were significantly affected by Omomer in absence of 4-OHT, indicating that the conditional form of the Omomyc protein is well tolerated.

Omomyc Induces Cytoskeletal Reorganization. Myc modulates expression of genes involved in cytoskeletal structure and cell adhe-

sion; in myoblasts, Myc transformation is characterized by cytoskeletal disorganization (11, 13). To determine whether Omomyc affected this aspect of cell transformation, Omomer was activated in Myc-C2C12 cells by the addition of 4-OHT and filamentous actin stained in fixed cells with fluorescent phalloidin. Dramatic cytoskeletal reorganization was observed in Myc-transformed cells on Omomer activation by tamoxifen, as indicated by the presence of actin stress fibers that are not visible in C2C12 cells expressing Myc alone (Fig. 1*B*).

Omomyc Potentiates Myc-induced Apoptosis. Differentiation into muscle tissue ensues when C2C12 cells are grown in 0.25% serum (differentiation medium). The process is blocked at a late stage in Myc-C2C12 cells, which are unable to form myotubes via cell fusion. Only modest cell death is observed under these conditions (11). To test whether Omomyc is able to restore myotube formation, C2C12 cells coexpressing Myc + Omomer were treated with 4-OHT and grown in differentiation medium. However, it proved impossible to determine the effect of Omomyc activation on myotube formation because all Myc + Omomyc cells underwent apoptotic death before any cell fusion could be observed. To investigate this cell death further, C2C12 cells coexpressing Myc + Omomer were stained with Hoechst-33258 dye and analyzed by fluorescence microscopy to identify any nuclei with morphological changes typical of apoptosis (Fig. 1*C*). In parallel, flow cytometric analysis was also conducted (Fig. 1*D*). After 12 h in differentiation medium, C2C12 cells infected with either control or Omomer retroviruses had comparable proliferation rates. Compared with C2C12 growing in normal medium, both showed some reduction in the fraction of S phase cells and an increased tendency to accumulate in G_1. In addition, a subdiploid, apoptotic cell population was present in Myc-C2C12 cells, which was much more evident in cells coexpressing Myc and Omomer. Hoechst staining was used to identify apoptotic nuclei at different time points

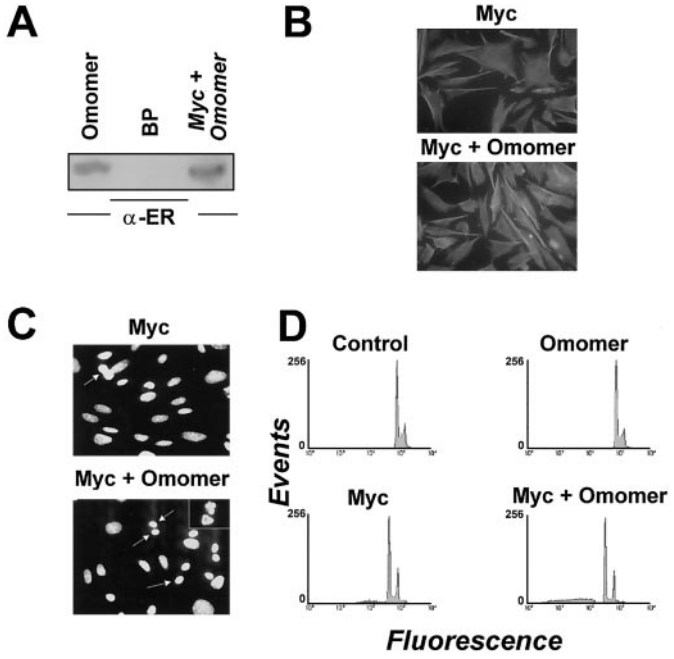

Fig. 1. Omomyc-induced cytoskeletal reorganization and increased apoptosis in C2C12 cells. *A*, omomer expression (Western blot) in C2C12 cells harboring control (*BP*), Omomer, or Myc and Omomer retroviruses (*Myc + Omomer*). *B*, actin fiber formation. Myc-C2C12 myoblasts harboring empty or Omomer retroviruses (*Myc* and *Myc + Omomer*, respectively) were stained with FITC-phalloidin. *C*, potentiation of Myc apoptosis. Myc and Myc + Omomer C2C12 cells, serum deprived and incubated with tamoxifen, were stained with Hoechst dye. Some nuclei displaying apoptotic morphological features are indicated by *arrows*. *D*, FACS analysis of serum-deprived cells. The subdiploid fraction, characteristic of apoptosis, represented 15% of the population in Myc cells and amounted to 25% in Myc + Omomer cells.

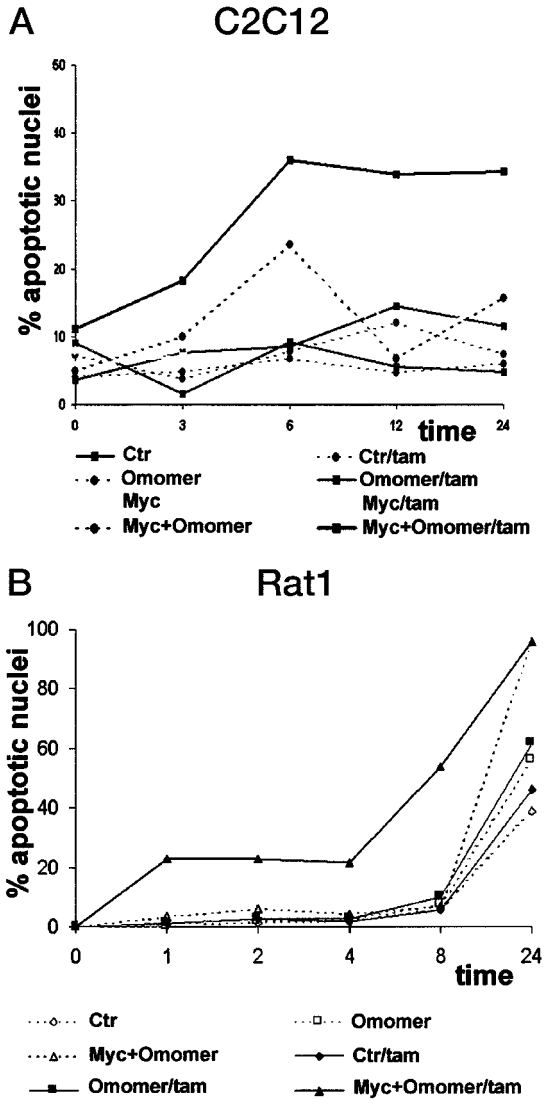

Fig. 2. Enhancement of apoptosis by Omomyc in different cell types. Apoptosis curves of C2C12 (*A*) and Rat1 (*B*) cells. Cells were cultured with or without 4-OHT (*tam*) and switched to a low-serum concentration. The percentage of apoptotic nuclei was determined by Hoechst staining. *Ctr*, wild-type cells with empty retrovirus; *Myc*, *Omomer*, *Myc + Omomer*, cells constitutively producing the indicated proteins.

after transfer into differentiation medium (Fig. 2*A*). In the absence of 4-OHT, Myc + Omomer-C2C12 myoblasts were indistinguishable from Myc-C2C12 infected with control virus. However, the extent of apoptotic nuclei was far greater after 4-OHT addition. Prolonged incubation in low serum, ≤48 h, resulted in the complete apoptosis of cells coexpressing Myc and Omomer. Most surprisingly, Omomer did not induce any additional apoptosis in the absence of overexpressed Myc (Fig. 2*A*). Thus, the capacity of Omomyc to induce apoptosis is dependent on Myc expression.

Analogous experiments were also performed on Rat1 fibroblasts, in which the Myc apoptotic response has been better characterized (14). Rat-1 cells were infected with Omomer retrovirus and Western blotting used to confirm Omomer expression. The cells were then reinfected with pBabeNeo-c/vMyc retrovirus and selected with neomycin. No apoptosis was observed in 10% serum growth medium, whereas serum deprivation (0.1%) induced dramatic apoptosis, starting after ~12 h. This rate was highly accelerated on 4-OHT activation of Omomer, in which case apoptosis was evident after as little as 1 h of serum deprivation and most evident within the first 8 h of serum deprivation (Fig. 2*B*). Omomer by

itself did not cause an apoptotic rate increase, confirming the Myc dependency of its proapoptotic effect.

Omomyc Interferes with Myc/Max Binding to the E-box. It is generally thought that all Myc functions require Myc/Max binding to the E-box (5). Because Myc/Omomyc heterocomplexes appear unable to bind to the E-box (8), we asked whether Myc E-box binding activity could be detected in extracts from cells coexpressing Myc and Omomyc using gel-shift assays on cell extracts (Fig. 3). As reported in Ref. 12, DNA binding of Myc/Max complexes was weakly detectable in control cells, whereas it was easily detected in cells overexpressing Myc. The Myc/Max signal disappeared on 4-OHT treatment of Myc + Omomer Rat1 cells, consistent with Omomyc inhibition of Myc binding to E-box DNA. Similar results were obtained with C2C12 cell extracts (data not shown).

Transcriptional repression may also contribute to the biological function of Myc (13, 15, 16). Unlike transactivation, gene repression by Myc appears not to depend on E-box binding. Rather, Myc repression seems to require both interaction with specific proteins, such as Miz-1 and Sp1, as well as recognition of specific DNA sequences that in certain instances have been associated with initiator elements (5, 17, 18). One possibility for the differential effect of Omomyc on Myc function is that Omomyc, while preventing transactivation at E-boxes, either has no effect or potentiates Myc transcriptional repression. In this way, Omomyc could direct Myc to a particular subset of target genes whose repression promotes apoptosis. To explore this possibility, we examined (Fig. 4) how Omomer activation affects expression of *cad* and *gadd45* genes, respectively, induced and repressed by Myc (19). Expression of *cad* mRNA is induced by Myc in exponentially growing Rat-1 cells, and this is attenuated significantly by coexpression of Omomyc. *gadd45* mRNA accumulates during serum deprivation, but its expression is rapidly repressed on induction of Myc after serum readdition. Expression of Omomyc had little effect on Gadd45 expression, although it may modestly enhance its repression (Fig. 4).

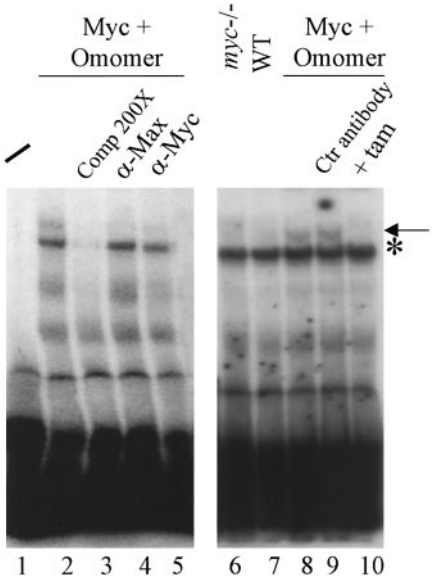

Fig. 3. Inhibition of Myc E-box binding by Omomyc. Gel shift assays with extracts from Rat1 cells coexpressing Myc and Omomer (*Myc + Omomer*, *Lanes 2–5* and *8–10*), Myc-null Rat1 cells ($myc^{-/-}$, *Lane 6*), and wild-type Rat1 (*WT*, *Lane 7*); Lane 1 has probe only. The Myc/Max-specific signal is indicated by an *arrow*; ∗, an ubiquitous complex, unrelated to Myc/Max (12). Samples were incubated with anti-Max (*Lane 4*), anti-Myc (*Lane 5*), a 200-fold excess of cold E-box probe (*Lane 3*), or control antibody (*Lane 9*). 4-OHT (*Lane 10*) caused disappearance of the Myc/Max signal.

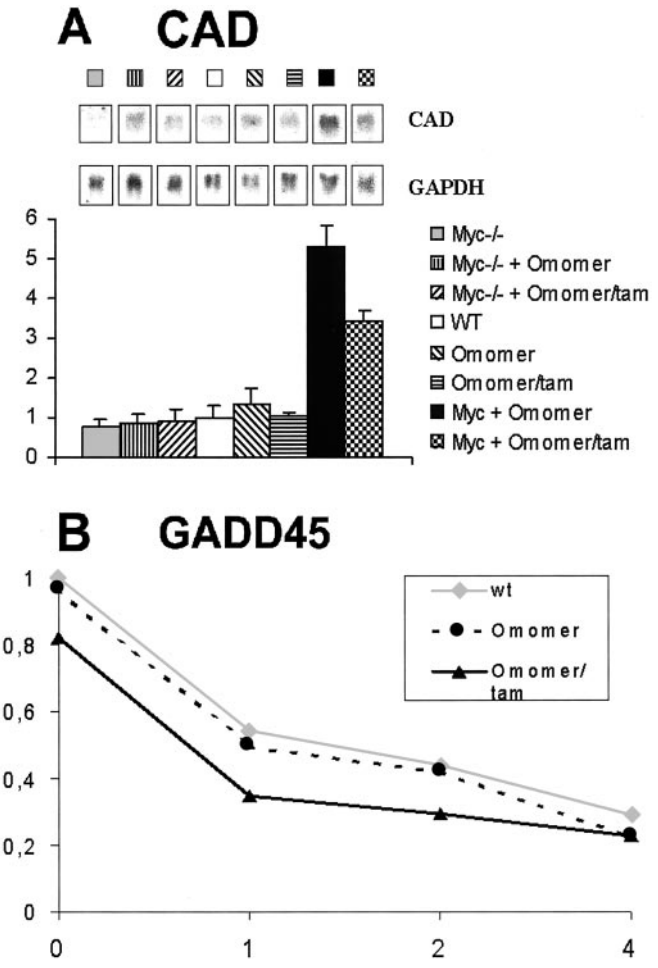

Fig. 4. Omomyc inhibits activation, but not repression, of Myc targets. Northern blot analysis of Cad (*A*) and Gadd45 (*B*) mRNAs in Rat1 cells exponentially growing or stimulated to re-enter the cell cycle, respectively. Signals are normalized to a glyceraldehyde-3-phosphate dehydrogenase mRNA standard and are relative to the value in wild-type cells; for Gadd45, the level before serum readdition was taken as a reference.

DISCUSSION

We have shown previously that the mutant bHLHZip Omomyc can interfere with artificial promoter activation by Myc/Max and impair proliferation in NIH3T3 cells. In this study, we asked whether expression of Omomyc also interferes with other known Myc functions. At first sight, Omomyc would appear to be a good Myc dominant negative, because it can interfere with Myc transformation of C2C12 myoblasts, reverting their cytoskeletal disorganization. However, we found that Omomyc exacerbates Myc-induced apoptosis. This proapoptotic activity is evident only in cells expressing elevated Myc, and we do not observe it in cells with endogenous c-Myc, which is typically expressed at very low levels. Thus, the capacity of Omomyc to promote apoptosis appears to depend on the level of Myc present. Moreover, Omomyc/Myc heterodimers appear not to bind the E-box. It has been reported that Myc transrepression, unlike transactivation, is E-box independent (5). Consistent with this, *cad*, a Myc known activated gene, is repressed by Omomyc. In contrast, Omomyc weakly enhances *gadd45* repression. It seems plausible that Myc/Omomyc complexes might substitute for, or modestly enhance, Myc/Max repressor function (Fig. 5), a notion consistent with observations that deletion of box I, in the NH_2-terminal part of c-Myc, diminishes transactivation without affecting Myc ability to accelerate apoptosis and that the ability of Myc-S, a natural Myc variant, to stimulate apoptosis in Rat1 cells correlates with its repressor activity (20–22). Mechanistically, one can envisage that Myc/Omomyc complexes retain

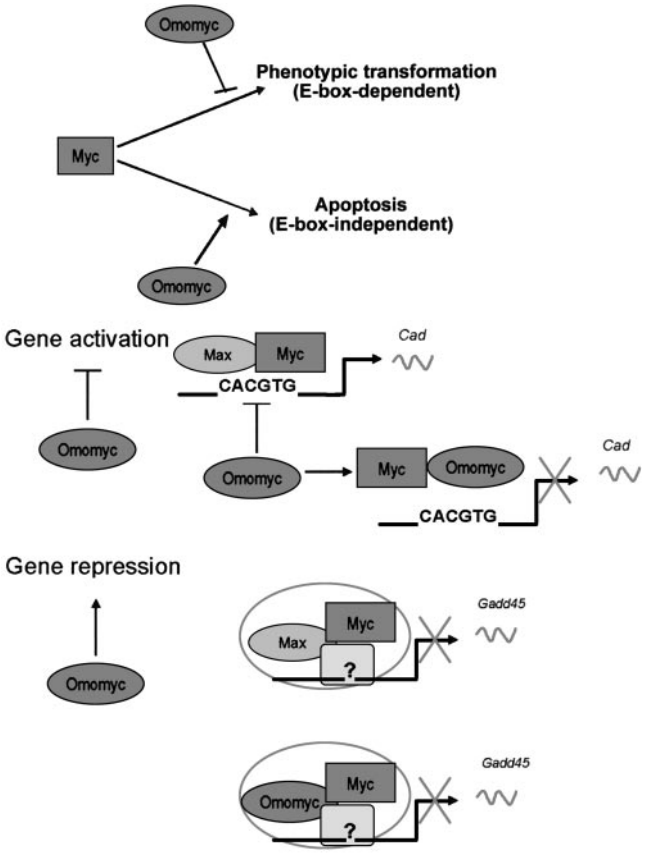

Fig. 5. Model depicting a dual role of Omomyc in Myc-regulated transcription.

the interactions with transcription factors binding different sites, such as the interaction reported between Miz-1 and the HLHZip domain of Myc, leading to $p15^{INK4b}$ promoter repression (17). An alternative explanation is that they have a role in chromatin modifications, by either binding to a component of the SWI/SNF chromatin remodeling complex or by influencing cofactor recruitment via the NH_2-terminal Myc box II, which is essential for apoptosis induction and interacts with TRRAP, Tip48, and Tip49 (7, 23–25).

Concerning the fact that C2C12 and Rat1 cell proliferation under growth promoting conditions was not very sensitive to Omomyc overexpression, the ability of Omomyc to impair colony formation, observed previously in NIH3T3 cells (8), is likely the result of a proapoptotic effect under the growth-limiting conditions of the colony formation assay rather than of an inhibition of cell cycle progression, even if the persistence of a residual Myc activity, still sufficient to drive proliferation of C2C12 and Rat1 cells, cannot be ruled out.

An alternative scenario is that Omomyc interacts with other Max-binding proteins, such as the Mad family members, thereby abrogating their putative survival function (26). However, we feel this interpretation is unlikely, because the Omomyc proapoptotic function in Rat-1 and C2C12 cells is clearly dependent on Myc overexpression. Moreover, we are unable to detect significant Mad expression by Northern analysis in conditions where Myc and Omomyc induce apoptosis (data not shown). The further possibility that Omomyc has additional molecular properties that result in cell killing seems inconsistent with our finding that Omomyc has no intrinsic apoptotic activity in the absence of Myc coexpression. Whatever the precise molecular mechanism, it is intriguing that Omomyc only induces apoptosis in cells in which Myc is already expressed.

Our findings suggest that it may be possible to enhance the intrinsic Myc apoptotic activity, and so influence tumor development or maintenance, by targeting the bHLHZip domain. It will be interesting to test whether this provides a novel therapeutic opportunity.

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