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The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max

B.Amati, T.D.Littlewood[1], G.I.Evan[1] and H.Land[2]

Growth Control and Development and [1]Biochemistry of the Cell Nucleus Laboratories, Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London, WC2A 3PX, UK
[2]Corresponding author

T.D.L. and B.A. contributed equally to this work

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The c-Myc protein (Myc) is involved in cellular transformation and mitogenesis, but is also a potent inducer of programmed cell death, or apoptosis. Whether these apparently opposite functions are mediated through common or distinct molecular mechanisms remains unclear. Myc and its partner protein, Max, dimerize and bind DNA *in vitro* and *in vivo* through basic/helix-loop-helix/leucine zipper motifs (bHLH-LZ). By using complementary leucine zipper mutants (termed MycEG and MaxEG), which dimerize efficiently with each other but not with their wild-type partners, we demonstrate that both cell cycle progression and apoptosis in non-transformed rodent fibroblasts are induced by Myc-Max dimers. MycEG or MaxEG alone are inactive, but co-expression restores ability to prevent withdrawal from the cell cycle and to induce cell death upon removal of growth factors. Thus, Myc can control two alternative cell fates through dimerization with a single partner, Max.

Key words: apoptosis/cell cycle/Max/Myc/oncogenes

Introduction

Activation of the c-*myc* proto-oncogene contributes to progression of a wide range of neoplasias, and generally leads to deregulated expression of the wild-type Myc protein. The oncogenic activity of c-*myc* can be demonstrated in transgenic animals or transfected cells in culture, and generally requires cooperation with at least one additional oncogene (reviewed in Luscher and Eisenman, 1990; Penn *et al.*, 1990c; Marcu *et al.*, 1992).

In non-transformed cells, Myc expression is tightly linked to mitogenic stimuli and is a prerequisite for cell growth (for reviews see Luscher and Eisenman, 1990; Penn *et al.*, 1990c; Marcu *et al.*, 1992). Moreover, post-translational activation of a Myc-oestrogen receptor chimera in resting cells is sufficient to induce entry into the cell cycle in the absence of other mitogenic stimuli (Eilers *et al.*, 1991). Consistent with this, deregulated expression of an exogenous c-*myc* gene in primary or established rodent fibroblasts renders them unable to exit from the cell cycle upon serum withdrawal. Instead, these cells continue cycling and concomitantly undergo apoptosis (Evan *et al.*, 1992). Expression of c-*myc* is also required for activation-induced apoptosis of T-cell hybridomas (Shi *et al.*, 1992), suggesting a role for Myc in this physiological process (for review, Evan and Littlewood, 1993).

The Myc polypeptide contains a basic/helix-loop-helix/leucine zipper (bHLH-LZ) domain which directs dimerization with the partner protein Max and sequence-specific DNA binding to the core hexanucleotide CACGTG (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991; Amati *et al.*, 1992, 1993; Berberich and Cole, 1992; Blackwood *et al.*, 1992b; Kato *et al.*, 1992; Littlewood *et al.*, 1992; Reddy *et al.*, 1992; Crouch *et al.*, 1993; Davis and Halazonetis, 1993; for reviews, Blackwood *et al.*, 1992a; Torres *et al.*, 1992; Evan and Littlewood, 1993). Consistent with its DNA binding specificity, Myc activates transcription of promoters containing CACGTG sites *in vivo* (Amati *et al.*, 1992; Benvenisty *et al.*, 1992; Kretzner *et al.*, 1992; Reddy *et al.*, 1992; Amin *et al.*, 1993; Crouch *et al.*, 1993; Gu *et al.*, 1993; Reisman *et al.*, 1993). Our studies with human proteins in yeast, which possesses no endogenous Myc and Max, demonstrated that Myc is inactive alone because it requires dimerization with Max to bind DNA (Amati *et al.*, 1992). *In vitro*, Myc alone neither forms homo-oligomers nor binds DNA, except at very high concentrations. In contrast, Max readily forms homodimers which bind to the same DNA sequence as Myc-Max (Amati *et al.*, 1992; Berberich and Cole, 1992; Littlewood *et al.*, 1992; Kato *et al.*, 1992; Ferré-d'Amaré *et al.*, 1993).

We previously developed a genetic complementation approach to demonstrate that the transforming activity of Myc is exerted by Myc-Max dimers (Amati *et al.*, 1993). We now report that induction of cell cycling and apoptosis by Myc in non-transformed cells also require interaction with Max.

Results

Genetic complementation approach to Myc-Max function

To analyse the biological activity of Myc-Max complexes, we designed dimerization specificity mutants that bind efficiently to each other but not to their wild-type partners. The mutants used in this work, MycEG and MaxEG, are shown in Figure 1, and have been previously described in detail (Amati *et al.*, 1993). Briefly, these mutants were designed by analogy with the solved crystal structure of a GCN4 LZ dimer (O'Shea *et al.*, 1991). This dimer folds as a parallel coiled-coil in which interhelical electrostatic interactions occur between the oppositely charged groups of residues positioned at specific positions (called g and e) of the LZ heptad repeat (Figure 1A). Four out of the six analogous g-e pairs in three contiguous heptad repeats of Myc and Max LZs contain oppositely charged residues (Figure 1B). Anticipating that these g-e pairs could be important determinants of dimerization specificity, we mutagenized *myc* and *max* cDNAs to exchange the residues

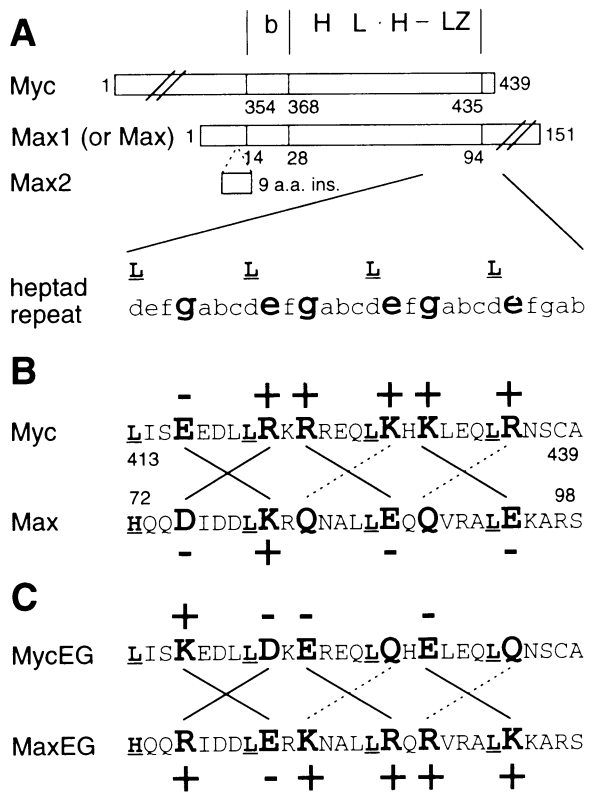

Fig. 1. Schematic representation of Myc, Max and of the LZ specificity mutants MycEG and MaxEG. (**A**) Wild-type Myc and Max proteins. Only the bHLH-LZ domains of all proteins (indicated at the top) are drawn to scale. Numbers indicate the amino acid residues at the end-points of the basic and HLH-LZ domains, and at the protein termini. Heptad repeat: general nomenclature of parallel coiled-coils (positions a–**g**) aligned with the LZ regions of (**B**) Myc and Max and (**C**) MycEG and MaxEG (amino acid sequences given in the one letter code). The periodic leucines are indicated by bold underlined characters. Residues at positions e and g of the heptad repeat (see text) are shown in larger font characters and their charge is indicated where appropriate. The solid lines in B and C indicate the potential electrostatic interactions between residues of opposite charges in Myc–Max and MycEG–MaxEG dimers, respectively. Dashed lines indicate putative interactions between glutamines (Q) and basic residues.

within each pair (Figure 1C). The resulting mutant proteins have reciprocally altered dimerization specificities: when expressed in cells, MycEG does not bind Max, and MaxEG does not bind Myc; only MycEG and MaxEG together form stable, biologically active dimers (Amati *et al.*, 1993).

Dimerization of Myc and Max is required for induction of cell cycle progression and apoptosis

To address the role of Max in Myc-induced cycling and apoptosis, we expressed MycEG and/or MaxEG in Rat1 fibroblasts by retroviral infection and compared the phenotypes of these cells with those of Rat1 cells which constitutively express human Myc (Rat1/Myc cells). Rat1/Myc cells fail to exit from the cell cycle following removal of mitogens, as indicated by the proportion of cells in S phase measured 48 h after serum withdrawal (Figure 2; Evan *et al.*, 1992). Concomitantly, these cells display extensive death by apoptosis in comparison with control cells (Figure 3A and B; Evan *et al.*, 1992). Cells that co-express the MycEG and MaxEG mutants display identical phenotypes (Figures 2 and 3C). Genomic DNA in the dying

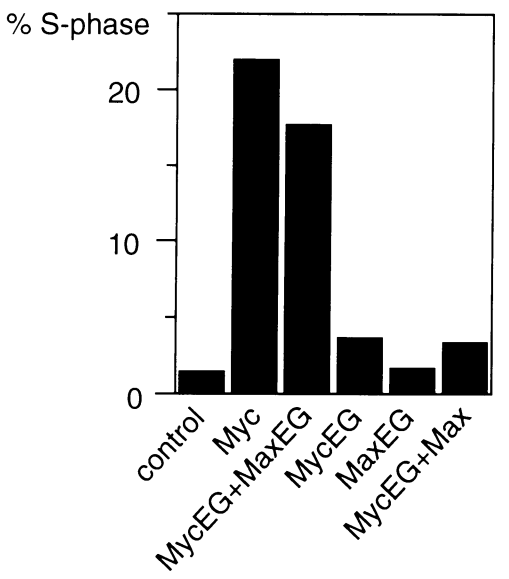

Fig. 2. Co-expression of MycEG and MaxEG prevents cell cycle arrest of Rat1 fibroblasts. Control cells (expressing no exogenous Myc or Max proteins) and cells expressing the indicated combinations of human Myc, Max, MycEG or MaxEG proteins were grown to confluence in medium with high serum (10%). The cells were then transferred to serum-free medium for 48 h, and the proportion of cells in S phase was determined. The data shown are from one of several independent experiments with similar results.

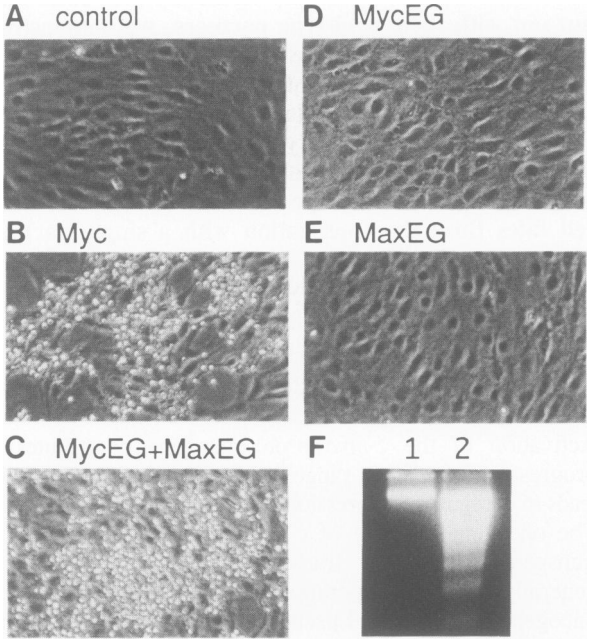

Fig. 3. Co-expression of MycEG and MaxEG induces apoptosis in Rat1 cells. (**A**–**E**) Control cells and cells expressing the indicated proteins were transferred from 10% serum to serum-free medium. Cells were examined by phase microscopy and photographed after 3 weeks. The kinetics of Myc-induced cell death depend on Myc expression levels (Evan *et al.*, 1992). In our experiments, using polyclonal populations of retrovirally infected cells, death of cells expressing Myc or MycEG and MaxEG occurs with similar kinetics and is readily seen after several days (data not shown). The prolonged incubation time in the experiment shown emphasizes the absence of cell death in the control population and in those expressing MycEG or MaxEG alone (A, D and E). No increase in cell death is observed when cells are grown in high serum (data not shown). (**F**) Agarose gel electrophoresis of DNA from control cells (lane 1) or from dying cells expressing MycEG and MaxEG (lane 2).

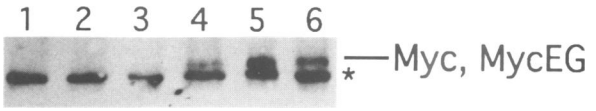

Fig. 4. Expression of MycEG and Myc in Rat1 cells. Lysates from retrovirally infected Rat1 cells expressing no exogenous protein (lane 2), MaxEG (lane 3), MycEG (lane 4), MycEG and MaxEG (lane 5) or Myc (lane 6) were analysed by immunoprecipitation followed by immunoblotting to visualize Myc and MycEG proteins as indicated. The band indicated by a star most likely corresponds to the reduced heavy chain of the Pan-Myc antibody used for the immunoprecipitations, which is spuriously recognized by the secondary HRP-RAM serum used in the immunoblotting (see Materials and methods). Indeed, this band also appears if cell lysate is omitted from the immunoprecipitation (lane 1).

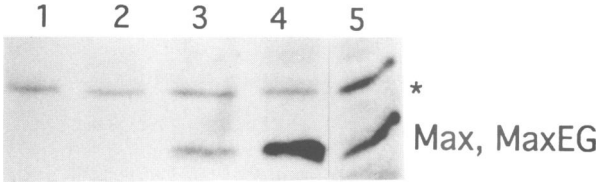

Fig. 5. Expression of MaxEG and Max in Rat1 cells. Lysates from retrovirally infected Rat1 cells expressing no exogenous protein (lane 1), MycEG (lane 2), MaxEG (lane 3), MycEG and MaxEG (lane 4) or MycEG and Max (lane 5) were analysed by immunoblotting to visualize Max and MaxEG proteins as indicated. 4×10^5 cell equivalents were loaded in each lane. The band indicated by a star is a non-specific, cross-reacting polypeptide of an apparent molecular weight of 29 kDa.

cells co-expressing MycEG and MaxEG is fragmented into nucleosomal fragments characteristic of apoptotic cells (Figure 3F), in a manner indistinguishable to that seen in serum-deprived Rat1/Myc cells (Evan *et al.*, 1992 and data not shown). Thus, MycEG and MaxEG together mimic wild-type Myc activity in Rat1 cells.

Both the enforced cycling and apoptotic phenotypes specifically require the formation of stable MycEG–MaxEG dimers *in vivo*, since neither is observed when either MycEG or MaxEG is expressed alone (Figures 2, and 3D and E). MycEG levels are comparable with Myc levels in Rat1/Myc cells, as determined by immunoprecipitation (Figure 4, lanes 4 and 6) or quantitative ELISA (Moore *et al.*, 1987; data not shown). Thus, the lack of phenotypic changes in Rat1/MycEG cells is due to the specific inability of MycEG to interact with Max, and not to low expression of the protein. Consistent with this, overexpression of the wild-type Max protein in Rat1/MycEG cells (Figure 5, lane 5) fails to rescue any biological activity (Figure 2 and data not shown). The mutant Max2EG, corresponding to a natural Max variant with a short amino-terminal insertion (Max2, Figure 1A) (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991), also rescues MycEG in these assays (data not shown). Taken together, these data show that the deficiency of MycEG in inducing cell cycling and apoptosis is rescued by the complementary mutants MaxEG or Max2EG.

It is noteworthy that in cells co-expressing MycEG and MaxEG, both proteins are found at higher levels than in cells expressing either alone (Figure 4, lanes 4 and 5, and Figure 5, lanes 3 and 4). This was observed in independently derived cell populations regardless of whether the MycEG- or MaxEG-coding retrovirus was introduced first (data not shown; see Materials and methods). We feel it most likely that this phenomenon is due to a selective growth advantage of the cells expressing active MycEG–MaxEG dimers, although we cannot rule out that dimerization may stabilize both proteins. This contention is supported by the observation that Rat1 cells that express active Myc display slightly higher growth rates and form denser colonies and monolayers in high serum than do control Rat1 cells or cells expressing either MycEG or MaxEG alone (data not shown).

We previously described two point mutations in the Myc basic region (360 N-P and 364/6/7R-A) that do not affect dimerization with Max but eliminate DNA binding (Amati *et al.*, 1992). Although the mutant proteins Myc360N-P and Myc364/6/7R-A are each efficiently expressed in cells infected with appropriate retroviruses, both are entirely deficient in inducing transformation, cell cycle progression or apoptosis (data not shown). We conclude that in addition to dimerization with Max, both the mitogenic and apoptotic activities of Myc most probably involve sequence-specific DNA binding.

Discussion

Our data demonstrate that dimerization with Max proteins is a prerequisite for Myc to induce apoptosis and cell cycle progression, measured as the maintenance of cells in the cycle after withdrawal of mitogens. The MycEG mutant fails to bind cellular Max proteins and is defective in these activities, which are restored by dimerization with the complementary mutant MaxEG. Thus, the apparently opposite physiological activities of Myc in cell cycle progression and apoptosis are both executed by Myc–Max dimers, and most likely through their interaction with specific DNA target sites.

In addition to its positive role, Max can also act as an antagonist of Myc function. Indeed, Max overexpression generally leads to an efficient, dose-dependent suppression of Myc activity in both cellular transformation and transactivation assays (Amati *et al.*, 1992, 1993; Kretzner *et al.*, 1992; Makela *et al.*, 1992; Mukherjee *et al.*, 1992; Prendergast *et al.*, 1992; Amin *et al.*, 1993; Gu *et al.*, 1993). This effect may be due to competitive displacement of Myc–Max dimers from DNA by Max either as a homodimer (Amati *et al.*, 1992), or as a heterodimer with the bHLH-LZ proteins Mad or Mxi-1 (Ayer *et al.*, 1993; Zervos *et al.*, 1993). However, whether Max–Max, Mad–Max or Mxi-1–Max dimers may antagonize cell cycle progression or apoptosis remains to be resolved.

The function of Myc–Max dimers as transcription factors (see Introduction) is believed to be relevant to their growth regulatory activities. The functionally identified transactivation domain of Myc (Kato *et al.*, 1990; Amati *et al.*, 1992; Kretzner *et al.*, 1992) co-maps with an amino-terminal region (NT) known to be essential for all tested biological activities of Myc (Stone *et al.*, 1987; Penn *et al.*, 1990b; Bar-Ner *et al.*, 1992; Evan *et al.*, 1992; Ohmori *et al.*, 1993; T.D.Littlewood, unpublished data). Deletion mutants of Myc that lack the NT domain but retain the bHLH-LZ fail to activate transcription of reporter genes (Amati *et al.*, 1992; Kretzner *et al.*, 1992) and also behave as strong dominant suppressors of Myc transforming activity (Mukherjee *et al.*, 1992; Amati *et al.*, 1993). Such mutants presumably form inactive dimers with Max that block binding sites on DNA and prevent transactivation of target

promoters. It has also been shown that the c-Myc NT has a greater potency than the L-Myc NT in both transactivation and transformation (Barrett *et al.*, 1992) and that overexpression of B-Myc, a protein homologous to the c-Myc NT but lacking a bHLH-LZ domain, appears to competitively inhibit both the transactivation and transformation activities of the c-Myc NT (Resar *et al.*, 1993). Thus, Myc–Max dimers most likely execute their biological functions by regulating the expression of specific target genes.

In fibroblasts, Myc-induced apoptosis, but not cell growth, can be counteracted by expression of the Bcl-2 protein (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992; Wagner *et al.*, 1993) or addition of specific cytokines, such as IGF-1 or PDGF (E.Harrington and G.I.Evan, unpublished data). This suggests that the apoptotic and mitogenic activities of Myc are exerted through distinct downstream pathways that can be differentially modulated by secondary signals (for review see Evan and Littlewood, 1993). In addition, neither the onset of apoptosis in Rat1/Myc cells nor its prevention by IGF-1 requires the synthesis of novel polypeptides (Evan *et al.*, 1992; E.Harrington and G.I.Evan, unpublished data). This implies that the components of the apoptotic pathway implemented by Myc are already present in growing cells, while cell death is suppressed as long as protective cytokines are present in the culture medium.

In summary, genes controlling the distinct pathways of cell cycle progression and apoptosis are regulated within the same Myc–Max-specific genetic programme. Whether common or distinct target genes are specifically involved in one or the other of these Myc-induced phenotypes remains to be established. This will be necessary to understand where the pathways governing the alternative cell fates of division and death diverge.

Materials and methods

Expression of Myc and Max proteins

Exogenous proteins were expressed in Rat1 cells by infection with retroviruses as previously described (Morgenstern and Land, 1990). The retroviral vector pDORhc-mycI,II,III expressing the wild-type human c-*myc* gene (Penn *et al.*, 1990a) and its mycEG derivative (Amati *et al.*, 1993), encode a neomycin resistance marker. The cDNAs encoding human Max (Blackwood and Eisenman, 1991) or MaxEG (Amati *et al.*, 1993) were subcloned into the vectors pBabeHygro or pBabePuro (Morgenstern and Land, 1990), encoding hygromycin and puromycin resistance markers, respectively. Cells expressing wild-type Myc were derived from a single infection. Those expressing MycEG + MaxEG, or MycEG + Max were derived from two successive infections, and those expressing MycEG or MaxEG alone were superinfected with parental retroviral vectors encoding the second drug resistance marker only. After each infection, drug resistant colonies were expanded as pools in medium containing 10% fetal calf serum and used for secondary infection or further experimentation.

Cell cycle analysis

To measure the fraction of cells in S phase, cells were pulse labelled with 10 μM bromodeoxyuridine for 1 h and 20 000 cells from each sample analysed in a fluorescence-activated cell sorter as previously described (Evan *et al.*, 1992).

Analysis of Myc and Max protein levels in infected Rat1 cells

To analyse Myc protein levels, cell lysates were prepared and equal amounts of total proteins (2 mg) subjected to immunoprecipitation as previously described (Littlewood *et al.*, 1992) with a rabbit Pan-Myc antiserum (Moore *et al.*, 1987). Immunoprecipitates were analysed by SDS–PAGE, immunoblotting and probing with the monoclonal antibody MycI-3C7 (Evan *et al.*, 1985), followed by a secondary peroxidase-conjugated rabbit anti-mouse serum (HRP-RAM, Dako) and visualization by chemiluminescence (ECL, Amersham).

Max proteins were analysed by direct immunoblotting analysis using the previously described MX antiserum (Littlewood *et al.*, 1992), followed by a secondary peroxidase-conjugated swine anti-rabbit serum (Dako) and ECL.

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