Brief Definitive Report

Functional Myc–Max Heterodimer Is Required for Activation-induced Apoptosis in T Cell Hybridomas

By Reid P. Bissonnette,* Anne McGahon,*‡ Artin Mahboubi*, and Douglas R. Green*

From the La Jolla Institute for Allergy and Immunology, La Jolla, California 92037; and the Cell Biology Laboratory, Department of Biology, St. Patrick's College, Maynooth, Ireland

Summary

T cell hybridomas respond to activation signals by undergoing apoptotic cell death, and this is likely to represent comparable events related to tolerance induction in immature and mature T cells in vivo. Previous studies using antisense oligonucleotides implicated the c-Myc protein in the phenomenon of activation-induced apoptosis. This role for c-Myc in apoptosis is now confirmed in studies using a dominant negative form of its heterodimeric binding partner, Max, which we show here inhibits activation-induced apoptosis. Further, coexpression of a reciprocally mutant Myc protein capable of forming functional heterodimers with the mutant Max can compensate for the dominant negative activity and restore activation-induced apoptosis. These results imply that Myc promotes activation-induced apoptosis by obligatory heterodimerization with Max, and therefore, by regulating gene transcription.

Ligation of the CD3 TCR complex on mature and immature T lymphocytes induces extensive gene expression, including the early induction of c-*myc*. In immature T cells and T cell hybridomas this activation also leads to apoptotic cell death in a manner which is dependent upon macromolecular synthesis (1–4). Previously, we used antisense oligodeoxynucleotides to inhibit the expression of c-Myc in activated T hybridoma cells. This treatment leads to a rapid loss of c-Myc protein and we found that this blocked activation-induced apoptosis (5). This and other studies (6–9) have implicated c-Myc in some forms of apoptosis. Thus, in addition to promoting cell entry and passage through the cell cycle, c-Myc functions to direct cells into an apoptotic pathway. The outcome of c-Myc activation is likely to depend upon other signals, such as those provided by growth factors (7, 8) or by other oncogenes (6, 10–12).

Myc is known to associate with several proteins within the cell, including the retinoblastoma protein (13), TFII-I (14), and Max (15, 16). This last protein forms a heterodimer with Myc which regulates gene transcription by binding to specific DNA sequences (17–20). We therefore sought to determine whether the role of c-Myc in activation-induced apoptosis was related to the functional activity of the Myc–Max heterodimer in control of gene expression. To investigate this, we have used dominant negative mutants of c-Myc and Max which have been demonstrated to block transcriptional regulation and transformation by c-Myc.

Materials and Methods

Plasmids, Cell Culture, and Transfections. The A1.1 T cell hybridoma has been previously described. Cells were maintained in IMDM supplemented with 5% FCS, 5×10^{-5} M β-mercaptoethanol, and 2 mM glutamine. The cells were transfected by electroporation using a capacitor-discharge pulse generator (GenePulser; Bio-Rad Laboratories, Richmond, CA). The cells were harvested from log phase cultures, suspended in the original conditioned medium at 10×10^6/ml and 0.8 ml cell suspension incubated for 15 min on ice in the presence of 20 μg linearized plasmid. The suspensions were pulsed once with 250 V at 960-μF capacitance in a 0.4-mm gap cuvette, and pulsed cells were incubated a further 15 min on ice, after which they were placed in 10 ml of fresh media. Transfected cell cultures were incubated for 24 h, washed, and then resuspended in fresh media. They were incubated an additional 10–14 d in the presence of selection media, containing either 800 μg/ml G418 (GIBCO BRL, Gaithersburg, MD) or 500 μg/ml hygromycin B (Sigma Chemical Co., St. Louis, MO). These bulk transfectants were reselected an additional 10 d in G418 or hygromycin before being used in the experiments described. Alternatively, individual clones were isolated by limiting dilution growth in the presence of selection antibiotics. The clones were screened for gene expression by reverse transcriptase-PCR (cDNA cycle; Invitrogen, San Diego, CA) using species- and construct-specific primers. All of the expression vectors containing the reciprocal exchange mutants or wild-type *myc* and *max* genes used in this study were constructed and described by Amati et al. (21). Briefly, the *myc* exchange mutant (mycRX) was constructed by replacing the helix-loop-helix leucine zipper (HLH/LZ) domain of c-Myc with the complementary domain from *max*. Both c-*myc* and mycRX were placed in a G418-selectable retroviral vector. The max exchange mutant (maxRX) and wild-type *max* were placed in a similar but hygromycin-selectable retroviral vector. All plasmids were generously provided by Dr. Bruno Amati (Imperial Cancer Research Fund, London, UK). All plasmids were linearized before transfections.

Induction and Analysis of Apoptosis. For the induction of apop-

tosis, the A1.1 T hybridoma cells were cultured for 16 h in plates precoated with the monoclonal anti-CD3ε antibody 145.2C11 (22) as previously described (2). For direct staining with propidium iodide (PI) to assess viability, the contents of each of triplicate culture wells were washed, resuspended in PBS containing 5 μg/ml PI, and analyzed immediately using a FACScan® (log scale) (Becton Dickinson & Co., Mountain View, CA). Dead cells were identified as those taking up the dye. For analysis by DNA content, the cells were washed once in PBS/5 mM EDTA and then fixed in 70% ethanol for 30 min at room temperature. The fixed cells were washed once in PBS and resuspended in PBS containing 100 μg/ml RNase A/200 U/ml RNase T1. The cells were incubated 15–30 min at room temperature, after which they were stained by adding 1 vol PBS containing 100 μg/ml PI. The cells were analyzed by FACScan® (linear scale) (Becton Dickinson & Co.). Apoptotic cells were identified by having less than 2 n DNA content (i.e., sub-diploid). Cells were assessed for DNA fragmentation in situ using a terminal deoxytransferase (TdT)-based nick end labeling assay which detects DNA strand breaks by using TdT to incorporate biotin-labeled UTP, which is subsequently detected by the addition of avidin-FITC (23). The cells were fixed in 1% formaldehyde/PBS, and stored in 70% ethanol. For UTP-biotin incorporation, the cells were resuspended in TdT buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 2.5 mM cobalt chloride, 0.25 mg/ml BSA, 100 U/ml TdT and 0.5 nM biotin-16-dUTP) and incubated for 30 min at 37°C. The cells were washed in PBS and resuspended in citrate/FITC-avidin buffer (0.6 M NaCl, 0.06 M NaCitrate, 2.5 mg/ml fluoresceinated avidin, 0.1% Triton X-100, 5% (wt/vol) nonfat dry milk). The cells were either analyzed immediately or fixed in PBS/1% formaldehyde for later analysis. All FACS® data analysis was performed using LYSYS II software. For the quantitation of apoptosis by acridine orange/ethidium bromide, cells were collected from cultures stimulated with anti-CD3 and 100 μl cell suspension immediately stained with 4 μl of a cocktail of 100 μg/ml acridine orange and 100 μg/ml ethidium bromide in PBS. Cells were scored as apoptotic/nonapoptotic by UV light microscopy.

Detection of Lymphokine Production. IL-2 production was determined by [^{3}H]TdR uptake of the IL-2-responsive cell line CTL.L (24) and quantitated using a recombinant IL-2 standard.

Results and Discussion

The strategy we used is illustrated in Fig. 1. A protein composed of Max with the HLH-LZ of Myc (MaxRX) forms a nonfunctional dimer with endogenous Max in the cell (21). This MaxRX–Max dimer acts as an inhibitor of normal Myc–Max heterodimer function in two ways: not only does MaxRX bind to and titrate out wild-type Max, the transcriptionally inactive Max–MaxRX dimer can bind to DNA and prevent binding of wild-type Myc–Max dimers (21). A reciprocal mutation in Myc, i.e., MycRX, exchanges the HLH-LZ of Myc for that of Max. However, this molecule might have only limited inhibitory activity, since although MycRX can bind efficiently to wild-type Myc, the MycRX–Myc dimer is not predicted to bind efficiently to DNA (21). However, when both the inhibitory MaxRX and the MycRX proteins are present, these will form a MycRX–MaxRX heterodimer that is functional. Studies by Amati et al. (21), upon which this strategy is based, showed that MycRX and MaxRX are both required for fibroblast transformation, neither one having transforming effects on its own.

Constructs encoding the mutant MaxRX protein were transfected into A1.1 T hybridoma cells and clones with stable expression were selected. Cells were then examined for their ability to undergo activation-induced apoptosis upon exposure to anti-CD3 antibody. As shown in the four experiments in Fig. 2, expression of MaxRX significantly inhibited anti-CD3–induced apoptosis, as determined by three different methods. In the first, cells were activated overnight and then stained with propidium iodide (without fixation) and analyzed by FACS® (Fig. 2 *A*). This simple method gives an accurate assessment of cell viability (since dead cells take up the dye [25]) but does not discriminate between apoptosis and necrosis. Alternatively, cells were activated, fixed, and stained with propidium iodide to examine DNA content. When analyzed by FACS®, apoptotic cells appear as a sub-

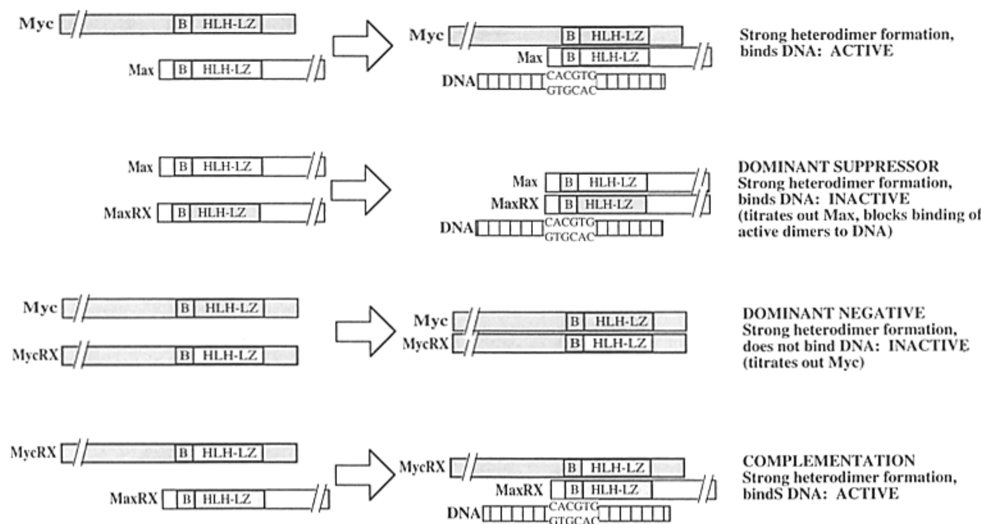

Figure 1. Schematic representation of the Myc/Max heterodimerization domain (HLH-LZ) exchange mutants used in this study and their relationships. Myc and Max form transcriptionally active heterodimers through the interaction of HLH-LZ motifs. MycRX (21) was generated by reciprocally exchanging the HLH-LZ domain of wild-type Myc with that of wild-type Max (RX for reciprocal exchange, Amati et al. [21]). MaxRX (21) was engineered by exchanging the HLH-LZ of wild-type Max with that from Myc. The diagram shows the interaction of these two exchange mutants, together with their interactions with the wild-type proteins. MycRX forms dimers with both MaxRX and with wild-type Myc; however, only MycRX–MaxRX dimers can bind DNA and are transcriptionally active. MaxRX can form dimers with MycRX or with wild-type Max. Either dimer may bind DNA, but only the MycRX–MaxRX dimer is active.

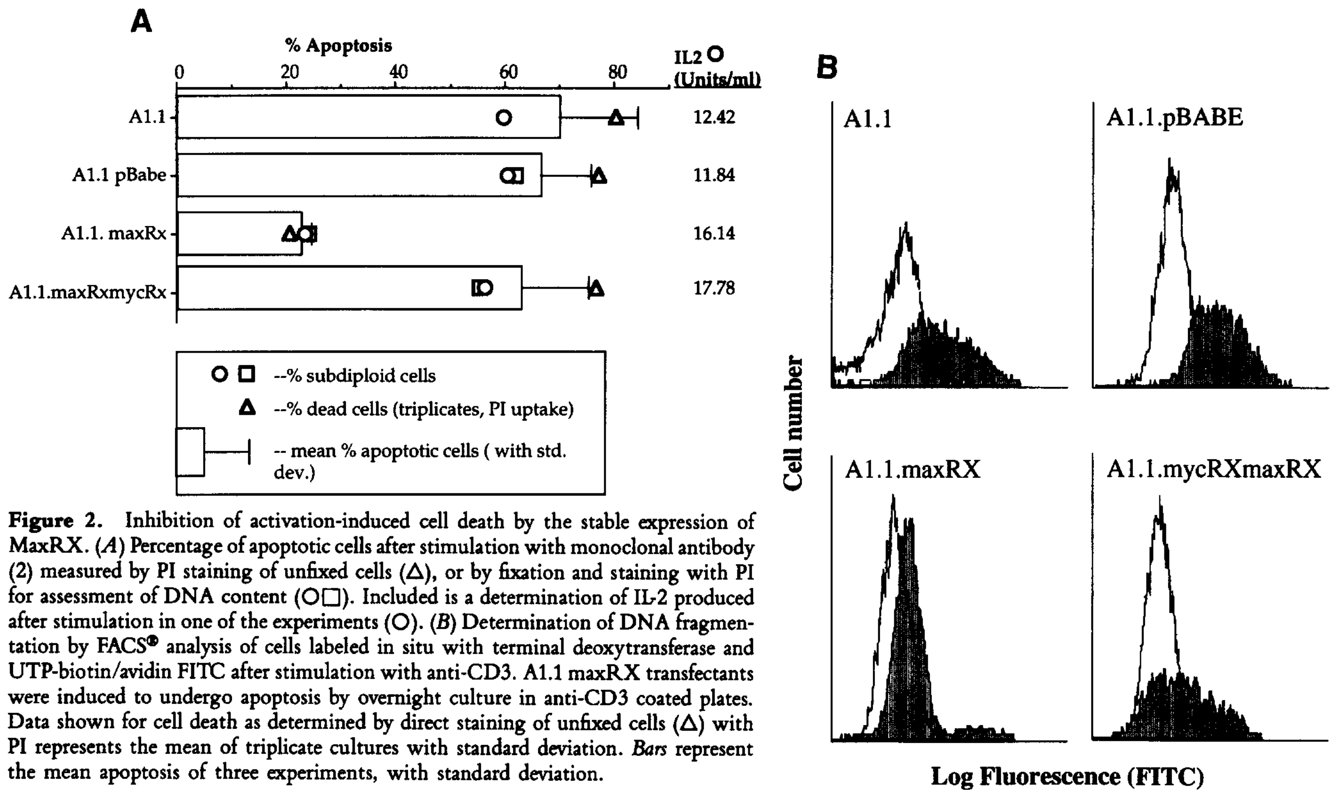

Figure 2. Inhibition of activation-induced cell death by the stable expression of MaxRX. (*A*) Percentage of apoptotic cells after stimulation with monoclonal antibody (2) measured by PI staining of unfixed cells (Δ), or by fixation and staining with PI for assessment of DNA content (O□). Included is a determination of IL-2 produced after stimulation in one of the experiments (O). (*B*) Determination of DNA fragmentation by FACS® analysis of cells labeled in situ with terminal deoxytransferase and UTP-biotin/avidin FITC after stimulation with anti-CD3. A1.1 maxRX transfectants were induced to undergo apoptosis by overnight culture in anti-CD3 coated plates. Data shown for cell death as determined by direct staining of unfixed cells (Δ) with PI represents the mean of triplicate cultures with standard deviation. *Bars* represent the mean apoptosis of three experiments, with standard deviation.

diploid population, owing to the fragmentation and subsequent loss of the soluble DNA fragments (Fig. 2 *A*) (26). Finally, cells were analyzed using an in situ terminal deoxytransferase reaction to label 3′-OH ends in the DNA, a method that detects apoptosis involving DNA fragmentation (23). As shown in Fig. 2 *B*, a pronounced apoptotic peak was observed after activation of control cells, but not cells expressing MaxRX. This effect of MaxRX was not observed in the presence of MycRX.

Our previous studies using c-*myc* antisense oligodeoxynucleotides indicated that inhibition of Myc expression in T hybridoma cells prevented activation-induced apoptosis but not activation-induced cytokine production (5). We therefore examined cytokine production in the cells expressing MaxRX. As shown in Fig. 2 *A*, cells expressing MaxRX produced a level of cytokine after activation that was equivalent to that of the other cells, despite significantly less activation-induced apoptosis. Thus, the function of the Myc–Max heterodimer appears not to be important to the expression of IL-2 after activation. Analysis of the 5′ flanking region of the IL-2 gene 2,300 bases upstream of the ATG start codon indicates the absence of a CACGTG consensus Myc–Max binding site.

The use of cloned cell lines generated after transfection and selection carries the potential problem that any clone might gain or lose a function due to random factors not related to the transfected gene. An alternative approach is to examine the effects of expression of these proteins in bulk. Thus, cells were transfected and selected, and their response to activation was examined without cloning. Results from several bulk transfections are shown in Fig. 3. Again, we observed that expression of MaxRX inhibited activation-induced apoptosis while coexpression of both MaxRX and MycRX did not. The effects of MycRX were inconsistent, but generally this molecule did not appear to significantly inhibit the induction of apoptosis. In one experiment we examined IL-2 production, and found equivalent levels of cytokine produced by cells expressing MaxRX, MycRX–MaxRX, and the control vectors, as above (results not shown). These results support those obtained using cloned cells above, and together, these demonstrate that in addition to Myc, the function of Max is required for activation-induced apoptosis. The ability of MycRX to block the inhibitory effects of MaxRX strongly suggests that Myc and Max function as a heterodimer in activation-induced apoptosis.

In these experiments we also examined possible effects of wild-type Max on activation-induced apoptosis in bulk transfected T hybridoma cells. Max is known to form homodimers (17, 18, 27, 28) and can inhibit the effects of the Myc–Max heterodimer in vivo (17, 20, 21, 29–31). As shown in Fig. 3, wild-type Max similarly inhibited activation-induced apoptosis. Thus, susceptibility to activation-induced apoptosis can apparently be regulated by the levels of Max in the cell, and this might therefore represent a potential control mechanism. Similarly, other proteins that influence the availability of Max (e.g., Mad [32]) might have roles in regulating activation-induced apoptosis.

In a number of cell types, Myc expression can trigger apoptosis (6–9) which is inhibited by growth factors (7, 8) or by the Bcl-2 protein (10–12). Most of these studies have involved the use of transfected cell lines. Recently, these observations were extended to group I Burkitt lymphoma cells which dis-

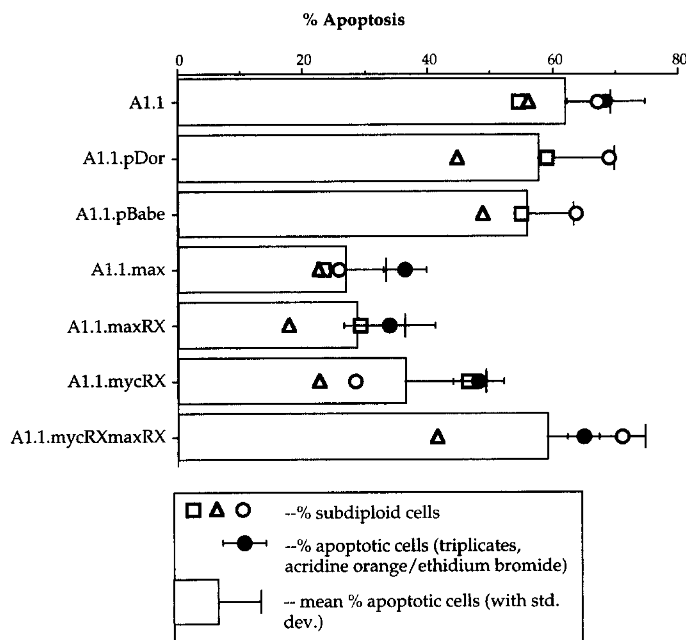

Figure 3. Inhibition of activation-induced apoptosis in bulk (uncloned) transfected cells expressing Myc and Max exchange mutants. Percent cells undergoing apoptosis after stimulation with monoclonal antibody to the TCR-CD3 complex as determined by FACS® analysis of DNA content in cells fixed and stained with PI (□△○), or by assessment of morphology and viability of cells in triplicate cultures stained with acridine orange/ethidium bromide, with standard deviation (●) (40). Bars represent the mean apoptosis of four experiments, with standard deviation. The induction and assessment of apoptosis by DNA content was as for Fig. 2 *A*. For the quantitation of apoptosis by acridine orange/ethidium bromide, cells were collected from cultures stimulated with anti-CD3 and immediately stained and scored by UV light microscopy. Data shown represents the mean of triplicate cultures with standard deviation.

play a high incidence of apoptosis in vitro and in vivo. Inhibition of c-Myc expression by IFN-α or c-*myc* antisense oligodeoxynucleotides protected these cells from apoptosis (33). Thus, c-Myc plays roles in apoptosis under a variety of conditions.

Amati et al. (34) have provided evidence that Myc-induced apoptosis in fibroblasts depends upon the interaction of Myc and Max. They used constructs encoding mutant proteins in which the residues forming electrostatic interactions within the leucine zipper heterodimerization motif were reciprocally exchanged. Thus, the resulting MycEG protein does not bind to Max, but does bind to a reciprocally modified MaxEG protein. When both are introduced into fibroblasts, the cells became highly susceptible to apoptosis. Thus, Myc induces apoptosis via interaction with Max. Our observations on activation-induced apoptosis are completely consistent with these findings.

In contrast to the systems described here, apoptosis may occur due to a decrease in Myc expression in some cases. Thulasi et al. (35) noted that glucocorticoids inhibit Myc expression in cells in which they induce apoptosis, and constitutive expression of Myc appears to protect these cells. Further, in a system partially related to the one described here, activation of the WEHI-231 B cell line via ligation of surface immunoglobulin induces apoptosis and a decrease in Myc expression (36). Interestingly, c-*myc*–antisense oligonucleotides were observed to stabilize expression of Myc in the activated B lymphomas and thereby prevent apoptosis. In support of this contrasting role for Myc as an apparent survival signal in the transformed B cells, lines that resist activation-induced apoptosis fail to downregulate Myc upon activation (37). Thus, Myc appears to have strikingly different roles in the induction versus prevention of apoptosis in different systems.

The Myc–Max heterodimer functions as a transcriptional regulator to induce gene expression. Our results and those of Amati et al. (34) in which Myc–Max promotes apoptosis therefore suggest that further transcription is required for cell death, and thus Myc–Max–induced genes should be examined for their potential roles in inducing apoptosis. One gene that is known to be induced by Myc is ornithine decarboxylase (ODC) (38). Recently, Packham and Cleveland examined the role of ODC in 32D cells, in which c-Myc expression promotes IL-3 withdrawal–induced apoptosis. They found that expression of the ODC gene from a constitutive promoter rendered 32D cells susceptible to apoptosis in the absence of exogenous Myc (39). Further, they found that pharmacologic inhibition of ODC activity in Myc-expressing cells reduced their susceptibility to apoptosis induction. Thus, at least one Myc-induced gene has been implicated in one form of apoptosis.

The authors would like to thank Dr. Bruno Amati for providing valuable reagents and Dr. Don Newmeyer for valuable discussions.

This work was supported by the National Institutes of Health (GM-52735) and by the American Cancer Society (CB-82).

Address correspondence to Reid P. Bissonnette, Ligand Pharmaceuticals, 9393 Towne Center Drive, San Diego, CA 92121, which is his current address.

Received for publication 22 June 1994.

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