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Inhibition of c-*myc* Expression Induces Apoptosis of WEHI 231 Murine B Cells

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Treatment of WEHI 231 immature B-lymphoma cells with an antibody against their surface immunoglobulin (anti-Ig) induces apoptosis and has been studied extensively as a model of B-cell tolerance. Anti-Ig treatment of exponentially growing WEHI 231 cells results in an early transient increase in c-*myc* expression that is followed by a decline to below basal levels; this decrease in c-*myc* expression immediately precedes the induction of cell death. Here we have modulated NF-κB/Rel factor activity, which regulates the rate of c-*myc* gene transcription, to determine whether the increase or decrease in c-Myc levels mediates apoptosis in WEHI 231 cells. Addition of the serine/threonine protease inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which blocks the normally rapid turnover of the specific inhibitor of NF-κB/Rel IκBα in these cells, caused a drop in Rel-related factor binding. TPCK treatment resulted in decreased c-*myc* expression, preventing the usual increase seen following anti-Ig treatment. Whereas inhibition of the induction of c-*myc* expression mediated by anti-Ig failed to block apoptosis, reduction of c-*myc* expression in exponentially growing WEHI 231 cells induced apoptosis even in the absence of anti-Ig treatment. In WEHI 231 clones ectopically expressing c-Myc, apoptosis induced by treatment with TPCK or anti-Ig was significantly diminished and cells continued to proliferate. Furthermore, apoptosis of WEHI 231 cells ensued following enhanced expression of Mad1, which has been found to reduce functional c-Myc levels. These results indicate that the decline in c-*myc* expression resulting from the drop in NF-κB/Rel binding leads to activation of apoptosis of WEHI 231 B cells.

The c-*myc* oncogene has been implicated in control of cell proliferation and differentiation as well as neoplastic transformation (17, 38). More recently, overexpression or inappropriate time of expression of the c-*myc* gene has been found to promote apoptosis. Askew and coworkers observed that addition of a vector expressing c-Myc protein accelerated apoptosis following interleukin-3 deprivation of the 32D interleukin-3-dependent myeloid cell line (6). Similarly, Evan et al. (22) found that transfection of 3T3 fibroblast cells with c-*myc* expression vectors led to enhanced levels of apoptosis upon growth arrest either by serum or isoleucine deprivation or by a thymidine block. The interaction of c-Myc with its binding partner Max (Myc-associated factor X) (12, 13) was found to be necessary to promote apoptosis (3). These findings have been further extended by using c-*myc* antisense oligonucleotides. Green and coworkers have shown that addition of these oligonucleotides to immature T cells and some T-cell hybridomas inhibited c-*myc* expression and prevented T-cell receptor-mediated apoptosis (48). Together these results strongly suggest that inappropriate overexpression of c-*myc* promotes apoptosis in some cell systems.

The WEHI 231 lymphoma cell line has been characterized as an immature B cell on the basis of surface markers and biological properties (43, 45). Growth of these cells can be arrested within 24 h by interaction with an antibody against the expressed surface immunoglobulin M (IgM) chains (14, 45). Boyd and Schrader (14) proposed this system as a model for self-induced B-cell tolerance. In support of this hypothesis, it was demonstrated that anti-IgM treatment causes apoptosis of WEHI 231 cells within 18 h (10, 27). Treatment of WEHI 231 cells with anti-IgM antiserum results in dramatic changes in c-*myc* expression. As we first showed several years ago, an initial 5- to 10-fold increase in c-*myc* mRNA levels between the first and second hours after treatment is followed by a rapid decline in c-*myc* expression (40). By 6 to 8 h of treatment, c-*myc* RNA and protein levels fall well below control values (36, 39). Several lines of evidence suggest that it is the drop in c-*myc* expression following anti-Ig treatment that results in apoptosis of WEHI 231 cells, in contrast to the findings discussed above. Binding of an introduced δ heavy chain (WEHI-δ) with an anti-IgD antibody resulted in an increase in c-*myc* RNA levels similar to that seen upon anti-μ treatment but not in the subsequent drop below the control levels (54). These cells do not cease proliferation, and apoptosis does not occur (54). Similar results were obtained by Ales-Martinez and coworkers with another B-cell line, CH33, also transfected to express IgD (2). Furthermore, mutants of WEHI 231 resistant to anti-IgM-induced apoptosis display only a transient increase in c-*myc* RNA levels following anti-Ig treatment (29), whereas treatment of WEHI 231 cells with transforming growth factor β (TGF-β) induces a drop in levels of c-*myc* expression and subsequently apoptosis (56). More recently, Fischer et al. (23) found that addition of an antisense oligonucleotide specific for c-*myc* prevented apoptosis of WEHI 231 cells but surprisingly led to stabilization of c-*myc* expression. Although the mechanism for this stabilization was not determined, these results also suggest that it is the drop in c-*myc* expression to levels below those seen in exponentially growing cells that signals apoptosis.

Recently we have demonstrated that the nuclear factor κB (NF-κB)/Rel family of transcription factors plays a major role in the control of c-*myc* gene expression following anti-Ig treatment of WEHI 231 cells (35). The murine c-*myc* gene contains

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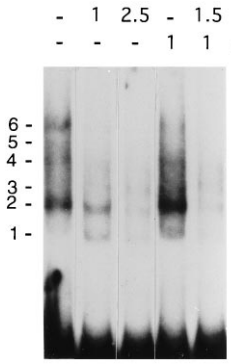


FIG. 1. TPCK treatment blocks activation of NF-κB/Rel binding in WEHI 231 cells. WEHI 231 cells were treated with 25 μM TPCK for 0.5 h and then incubated in the presence or absence of anti-Ig for an additional 1 h. Alternatively, cells were treated with 25 μM TPCK alone for 1 or 2.5 h. Nuclear extracts were prepared from control and treated cells, and equal samples of protein (5 μg) were used in electrophoretic mobility shift analysis with a DNA fragment containing the upstream NF-κB element from the murine c-*myc* gene. The specificities of the changes were confirmed by mobility shift analysis with an AP-1 oligonucleotide (data not shown). Identities of most of the bands, labelled 1 through 6, were determined previously by using specific antibody reagents (35) and are as follows: band 1, p50 homodimer; band 2, p50–c-Rel; band 3, p50-RelA; band 4, p50 and unidentified subunit; and band 5, c-Rel homodimer. Band 6 was unidentified.

two functional NF-κB elements, which we termed the upstream regulatory element (URE) and the internal regulatory element (IRE) (20, 33). Exponentially growing WEHI 231 cells express a large amount of nuclear NF-κB/Rel factors, in part because of the rapid rate of turnover in these cells of the specific inhibitory protein IκBα (42, 46). We have recently demonstrated that changes in the binding of NF-κB/Rel factors mediate signals leading to both the increase and the decrease in c-*myc* expression following anti-Ig treatment (35). To test directly whether it is the increase or the decrease in c-*myc* expression that promotes apoptosis of WEHI 231 cells, here we have used the protease inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which inhibits IκBα turnover in WEHI 231 cells (28, 42), to modulate NF-κB/Rel activation and binding. Addition of TPCK led to a rapid drop in levels of NF-κB/Rel binding that could not be overcome by anti-Ig treatment. TPCK blocked the increase in c-*myc* expression normally resulting from anti-Ig treatment and, when added alone, caused a decrease in c-*myc* expression. TPCK induced apoptosis when added alone and failed to block apoptosis mediated by anti-Ig. Ectopic expression of c-Myc decreased levels of apoptosis induced by TPCK or anti-Ig treatment. Furthermore, to reduce functional c-Myc levels, cells were microinjected with the Max binding partner Mad1 and apoptosis was induced. These results indicate that the drop in c-*myc* expression following TPCK treatment plays a pivotal role in the induction of cell death in the WEHI 231 B-cell line.

MATERIALS AND METHODS

Culture and treatment conditions. WEHI 231 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 0.35% glucose, 0.058% glutamine, nonessential amino acids (Gibco Laboratories, Grand Island, N.Y.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μM β-mercaptoethanol, as described previously (35). Prior to treatment, cells were diluted to a density of 4×10^5 cells per ml with fresh warm medium and allowed to incubate for a minimum of 4 to 5 h. Cell cultures were incubated with 10 to 25 μM TPCK (Sigma Chemical Co., St. Louis, Mo.) for the indicated times. Cells were treated with 1:1,000 dilution of anti-μ heavy chain antibody (Cappel Laboratories, Cochranville, Pa., or Calbiochem, La Jolla, Calif.) for 1 to 52 h.

Electrophoretic mobility shift analysis. Crude nuclear extracts were prepared by the method of Strauss and Varshavsky (53). The 221-bp fragment of DNA spanning *Bgl*II to *Acc*I, including bp −1139 to −921 relative to the P1 promoter of the murine c-*myc* gene, was subcloned into pUC19. This DNA, termed fragment A previously (20), includes the URE κB element, which is located 1,101 to 1,081 bp upstream of the P1 promoter. DNA fragments were end labelled with the large fragment of *Escherichia coli* DNA polymerase I (New England Biolabs) and α-^{32}P-deoxynucleoside triphosphates (New England Nuclear). The electrophoretic mobility shift assay (EMSA) was performed as follows. Each ^{32}P-labelled fragment (25,000 cpm; approximately 2 ng) and 3.5 μg of nuclear extract were mixed in 70 mM NaCl–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5)–1 mM EDTA–1 mM dithiothreitol–0.1% Triton X-100–4% (vol/vol) glycerol–5 μg of poly(dI-dC) · poly(dI-dC) copolymer in a final volume of 25 μl. This mixture was incubated for 30 min at 22°C. Gels were electrophoresed at 11 V/cm in a 4 or 4.5% polyacrylamide gel with TAE running buffer (6.7 mM Tris-HCl [pH 7.5], 3.3 mM sodium acetate, 1 mM EDTA), dried, and subjected to autoradiography.

Plasmids and transfection. The p1.6 Bgl chloramphenicol acetyltransferase (CAT) construct contains bp −1141 to +513 of the murine c-*myc* gene, containing promoter-upstream-exon 1 sequences, including both the NF-κB upstream and internal regulatory elements (21). The double-mutant p1.6 Bgl construct (dm p1.6 Bgl), prepared by the double-primer site-directed mutagenesis method, contains two G-to-C transversions in both the URE and the IRE, preventing Rel factor binding and transactivation (21, 34).

For transient transfections, exponentially growing WEHI 231 cells were washed once with medium and resuspended in Dulbecco modified Eagle medium supplemented with 20% fetal calf serum at a concentration of 20×10^6 cells per ml. Cells (250 μl) were preincubated on ice for 10 min with DNA (40 μg). Cells were transfected by electroporation at 240 V and 960 μF using a Gene Pulser (Bio-Rad Laboratories). Following incubation on ice for 5 min, the cell suspension was mixed with 1.75 ml of complete medium and incubated for 10 min at room temperature. The suspensions were transferred to petri dishes, and some were further treated with TPCK and/or anti-Ig. Following incubation at 37°C for 8 h, cells were harvested and the resulting extracts were normalized for total protein content by using the Bio-Rad protein quantitation kit. Equal amounts of lysates were assayed in duplicate for CAT activity, as we have described previously (35). Standard deviations were obtained by Student's *t* test.

Linearized vector DNA was used to isolate stable transfectants expressing the pRc-CMV-myc vector (25). DNA (30 μg) was incubated with *Xba*I, and then the enzyme was inactivated by incubation for 5 min at 68°C. Alternatively, 30 μg of pSV2-neo was used. For preparation of the pM21 c-*myc* expression vector (9), stable transfectants were prepared by using 40 μg of pM21 and 2 μg of pSV2-neo. Electroporation was performed essentially as described above. After 24 to 48 h, 1.2 mg of G418 (Gibco Laboratories) per ml was added to the culture medium and selective growth conditions were maintained for approximately 2 weeks. Clones were isolated by limiting dilution.

RNA and protein analysis. Total RNA was isolated with TRI reagent (Mo-

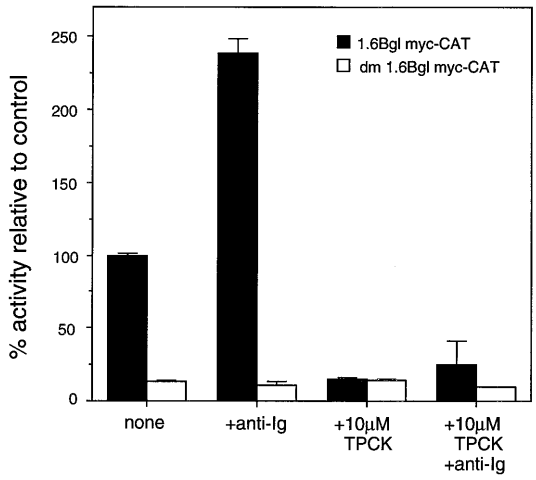


FIG. 2. TPCK inhibits transcription of the c-*myc* promoter. WEHI 231 cells were transfected with 40 μg of DNA of a p1.6 Bgl wild-type CAT construct or a double-mutant (dm) construct in which both NF-κB elements contain two G-to-C conversions such that Rel factor binding is prevented. Following transfection, cultures were divided and treated with anti-Ig in the presence or absence of 10 μM TPCK, as indicated. Alternatively, cells were treated with 10 μM TPCK alone. Extracts were prepared after 7 h and assayed for CAT activity. The data are presented relative to untreated wild-type p1.6 Bgl CAT activity, which is set at 100%. Standard deviations were obtained by Student's *t* test.

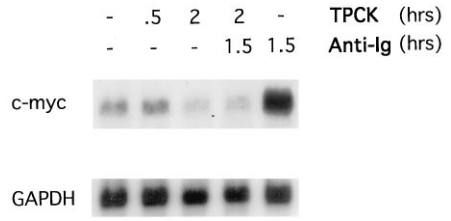


FIG. 3. TPCK blocks activation of c-*myc* RNA expression. WEHI 231 cells in exponential growth were treated with 25 μM TPCK for 30 min and then incubated in the absence or presence of anti-Ig for an additional 1.5 h. Alternatively, cells were treated with anti-Ig alone for 1.5 h. Total RNA was isolated from untreated and treated cells, and samples (15 μg) were subjected to Northern blot analysis for RNA expression of c-*myc* or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as a control for equal loading.

lecular Research Center, Inc., Cincinnati, Ohio) according to the manufacturer's protocol. Samples of RNA (15 μg per lane) were analyzed by Northern (RNA) blotting, as we have described previously (36). Probes used included the mouse c-*myc* cDNA clone pM-c-*myc*54 (51) and a glyceraldehyde-3-phosphate dehydrogenase clone (19).

Nuclear proteins were isolated by using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 1% sodium sarcosyl) as described previously (4). The protease inhibitors leupeptin (10 μg/ml), phenylmethylsulfonyl fluoride (0.5 mM), and dithiothreitol (1 mM), which were prepared as fresh stocks, were added to buffers just prior to use. The nuclear suspension was sheared and the nuclear debris was removed via centrifugation, and the supernatant was subjected to SDS–10% PAGE analysis with Rainbow markers (Amersham) and processed for Western blotting (immunoblotting), as we have described previously (39) except that membranes were developed with a Du Pont chemiluminescence kit (NEN). An affinity-purified antibody (50-23) against whole mouse c-Myc protein (kindly provided by S. Hann, Vanderbilt University, Nashville, Tenn.) was used at a 1:1,000 dilution.

Apoptosis assays. For analysis of DNA laddering, about 10^6 cells were used according to the procedure of Smith et al. (50). Briefly, control or treated cells were collected by centrifugation and washed in cold phosphate-buffered saline (PBS) twice. Pellets were then resuspended in 20 μl of lysis buffer containing 10 μM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5% (wt/vol) sodium laurylsarcosine (Sigma), and 0.5 mg of proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) per ml, and the mixture was incubated at 50°C for 1.5 h. Then 10 μl of RNase A (0.5 mg/ml) was added, and the incubation was continued for 1 h. Samples were then heated to 70°C for 10 min, quickly chilled, and mixed with 10 μl of loading buffer consisting of 1% low-melting-point (LMP) agarose (Bethesda Research Laboratories, Gaithersburg, Md.), 40% (wt/vol) sucrose, and 0.25% (wt/vol) bromophenol blue prior to electrophoresis in a 1.5% agarose gel. The 123 ladder DNA (Gibco) was used as a marker. Electrophoresis was performed overnight at 20 V in 1× TAE buffer.

For the fluorescence assay for apoptosis, quantitation of apoptotic cells was performed as previously described (26, 30). Briefly, cells were washed in cold PBS, pelleted, and resuspended in 1.5 ml of hypotonic fluorochrome solution containing 5.0 μg of propidium iodide (PI) (Sigma Chemical Co.) per ml, 1% sodium citrate, and 0.1% Triton X-100 (Sigma). Treated cells were analyzed on a Becton Dickinson FACScan flow cytometer. Cells undergoing DNA fragmentation and apoptosis were shown to be weaker in PI fluorescence than those in the typical G_0/G_1 cell cycle.

For trypan blue exclusion assays, cells were incubated with 0.2% trypan blue (Gibco) for 10 to 20 min, except where noted, and the percentage of cells excluding dye (viable cells) or staining positive (dead cells) was determined. Phase-contrast images were obtained at a magnification of ×100 by using Technical Pan film (ASA 25). The Non-Radioactive Cell Proliferation assays (Promega, Madison, Wis.) were performed in 100-μl volumes in 96-well dishes. For each control and treated assay point, 4×10^4 cells per well for anti-Ig or 6×10^4 cells per well for TPCK were incubated either in duplicate or in triplicate for 3 h at 37°C with (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) solution (333 μg/ml) and 25 μM phenazine methosulfate (PMS) according to the manufacturer's directions. The A_{490} was measured in an enzyme-linked immunosorbent assay (ELISA) plate reader. For controls, medium without cells was similarly incubated to zero the reaction.

Microinjection analysis. WEHI 231 cells were allowed to attach to tissue culture plates in the presence of culture medium containing 0.4% fetal bovine serum (FBS) and supplemented with 20 mM HEPES, pH 7.3. After 30 min of incubation at 37°C, all cells in a circled area were microinjected with the indicated protein at 1 mg/ml in 130 mM KCl–10 mM sodium phosphate (pH 7.3) by using a Narishige micromanipulator and glass capillaries (tip diameter, 0.1 μm) under conditions of constant nitrogen flow at a pressure of 0.4 lb/in^2. Either Mad1–glutathione *S*-transferase (GST) protein (8) or control GST protein was prepared as we have described previously (4) and concentrated by spin dialysis using Ultrafree CL filters (Millipore, Marlborough, Mass.) to a concentration of 1 mg/ml for microinjection. Alternatively, 1 mg of Mad1 expression vector (pMAD.VZ1; kindly provided by R. DePinho [Albert Einstein College of Medicine, Bronx, N.Y.]) per ml was microinjected in the absence or presence of a pM21 c-Myc expression vector (1 mg/ml). Following microinjection, cloning rings were placed over the microinjected areas and the medium was replaced with 10% FBS–Dulbecco modified Eagle medium. After 30 min of incubation at 37°C, cells that had survived the microinjection were detached from the tissue culture plates by gentle trituration, transferred to multiwell plates, and incubated at 37°C. After 20 h, 1/10 of a volume of a trypan blue solution (0.04% final concentration) was added to each well, cells were incubated for 15 min, and viable cell counts were obtained. Alternatively, cells were fixed at 4.5 h after microinjection in 3.7% formaldehyde in 0.2× PBS for 10 min at room temperature and then transferred to a glass slide. After air drying, cells were rehydrated in 1× PBS and stained in 5 μg of PI per ml for 15 min at room temperature. After mounting in 90% glycerol–PBS, cells were viewed in a Nikon Optiphot microscope and fluorescent images were recorded at a magnification of ×200 by using TMAX (ASA 3200) film.

RESULTS

TPCK reduces NF-κB/Rel factor binding activity. To determine whether treatment with the protease inhibitor TPCK, which stabilizes IκBα in WEHI 231 (28, 42; also data not shown) down-regulates NF-κB/Rel binding, cells were incubated in the absence or presence of 25 μM TPCK for 0.5 h and then subsequently treated with anti-Ig for 1 h. Alternatively, cells were incubated in TPCK alone for 1 or 2.5 h. Nuclear extracts were prepared, and an EMSA using the 221-bp *Bgl*II-to-*Acc*I DNA fragment upstream of the c-*myc* promoter, which contains the upstream NF-κB element, was performed (Fig. 1). Extracts from untreated exponentially growing WEHI 231 cell nuclei contain six distinguishable specific NF-κB/Rel complexes (20, 35) (see the legend to Fig. 1). Treatment with TPCK alone significantly decreased the levels of NF-κB/Rel complexes 2 through 6 by 1 h, and the levels continued to decrease with time. TPCK significantly reduced formation of RelA- and c-Rel-containing complexes, including p50-RelA (band 3), p50–c-Rel (band 2), and c-Rel homodimers (band 5). In contrast, formation of p50 homodimer (band 1) was only modestly affected. Thus, TPCK treatment resulted in decreased formation of complexes that transactivate either modestly (p50–c-Rel) or potently (p50-RelA), but it has less effect on p50 homodimers which are unable to transactivate c-*myc* in vivo (34, 35). Furthermore, the presence of TPCK caused a

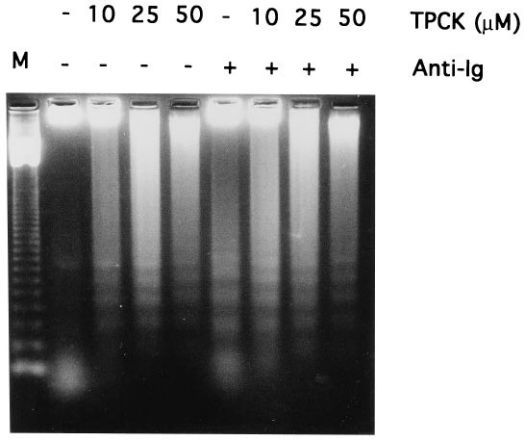


FIG. 4. TPCK treatment induces apoptosis in WEHI 231 and fails to prevent anti-Ig-mediated apoptosis. WEHI 231 cells in exponential growth were pretreated with 0, 10, 25, or 50 μM TPCK for 0.5 h and then incubated in either the presence or the absence of anti-Ig. After 13 h, cultures were subjected to DNA laddering assay as a measure of induction of apoptosis. M, 123 marker DNA.

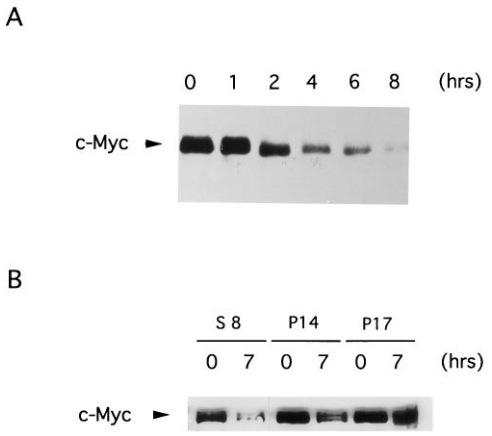


FIG. 5. Levels of c-Myc protein remain elevated following TPCK treatment of pM21 c-*myc*-transfected lines. (A) TPCK treatment reduces expression of c-Myc protein in parental WEHI 231 cells. Nuclear proteins were extracted from WEHI 231 cells in exponential growth (0 h) or following treatment with 25 μM TPCK for 1, 2, 4, 6, or 8 h and subjected to immunoblotting using an affinity-purified c-Myc antibody prepared against the whole murine c-Myc protein. (B) Maintenance of c-Myc expression in pM21-transfected lines. Nuclear proteins were prepared from WEHI 231 stable transfectants S8-WEHI 231 (pSV2neo) and P14- and P17-WEHI 231 (pM21 c-*myc* expression vector) in exponential growth (0 h) or following 7 h of treatment with 25 μM TPCK and analyzed for c-Myc protein expression as described for panel A.

similar drop in binding in cells treated with anti-Ig, preventing the increase in formation of complexes 2 and 3 normally seen following 1 h of treatment with anti-IgM. These results suggest that stabilization of the normally labile IκBα protein causes a rapid decrease in nuclear binding of NF-κB/Rel complexes and prevents the increase in binding that normally follows anti-Ig treatment.

TPCK suppresses the transcriptional activity of the c-*myc* promoter. To monitor the effects of TPCK treatment on c-*myc* gene transcription, transfection analysis was performed. The p1.6 Bgl construct consists of bp −1141 to +513 of the murine c-*myc* gene, containing promoter-upstream-exon 1 sequences, including both the upstream and internal NF-κB elements, linked to the CAT reporter gene (21). The double-mutant p1.6 Bgl construct (dm p1.6 Bgl) contains two G-to-C transversions in both the URE and the IRE. Following introduction of these constructs into WEHI 231 cells via electroporation, cells were incubated for 7 h with anti-Ig either in the absence or in the presence of TPCK (10 μM) or with TPCK alone (Fig. 2). Anti-Ig treatment induced the CAT activity of the wild-type p1.6 Bgl construct, but not the mutant construct, approximately 2.5-fold, consistent with previous findings (35). TPCK treatment alone decreased activity of the wild-type construct 6.6-fold compared with the control. The levels were reduced to that of dm p1.6 Bgl, consistent with NF-κB/Rel binding modulating basal c-*myc* gene transcription. Addition of TPCK to anti-Ig-treated WEHI 231 cells not only prevented the increase in CAT activity but resulted in a fourfold drop relative to basal levels, consistent with the effects on NF-κB/Rel binding observed above. These findings suggest that c-*myc* expression is down-regulated at a transcriptional level by TPCK, as expected on the basis of the drop in NF-κB/Rel binding.

TPCK treatment reduces the basal level of c-*myc* mRNA and prevents the normal induction following anti-Ig treatment. To monitor the effects of TPCK on c-*myc* RNA levels, RNA was isolated from untreated WEHI 231 cells or following pretreatment with 25 μM TPCK for 30 min and then incubation with anti-Ig for 1.5 h. Alternatively, cells were incubated with TPCK

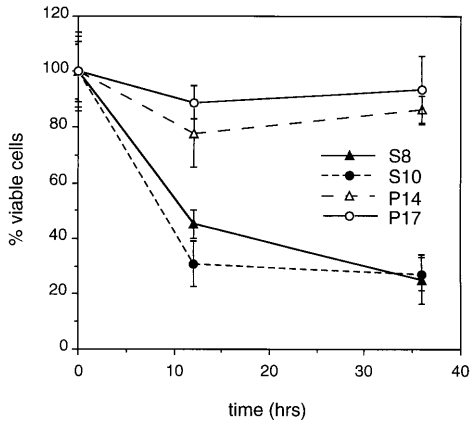


FIG. 6. Ectopic expression of c-Myc reduces cell death following TPCK treatment. Stable WEHI 231 clones transfected with either pSV2neo (S8- and S10-WEHI 231) or pM21 c-*myc* expression vector (P14- and P17-WEHI 231) were treated in triplicate with 25 μM TPCK for 12 or 36 h and analyzed for cell viability by conversion of MTS dye to its formazan product. Data are plotted as percent viable cells relative to untreated control cultures.

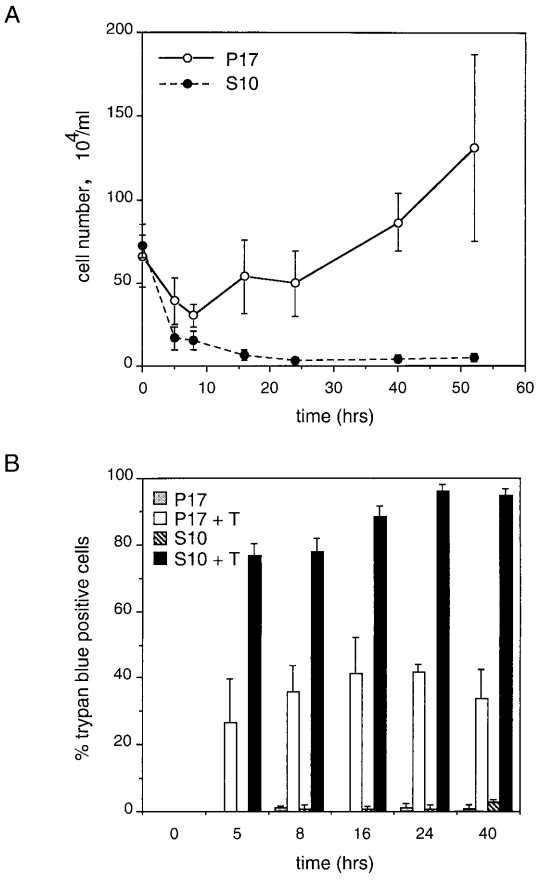


FIG. 7. P17-WEHI 231 cells, ectopically expressing c-Myc, display extensive cell survival following TPCK treatment. Cultures of clones S10-WEHI 231 (pSV2neo) and P17-WEHI 231 (pM21) were incubated in triplicate in the absence or presence of 25 μM TPCK, and trypan blue-positive and -excluding cell numbers were determined. (A) Cell survival curve. Numbers of cells excluding trypan blue are plotted as a function of hours of treatment with TPCK. (B) Induction of cell death. The time course of appearance of trypan blue-positive cells in control, untreated cultures and TPCK-treated cultures (+ T) is shown.

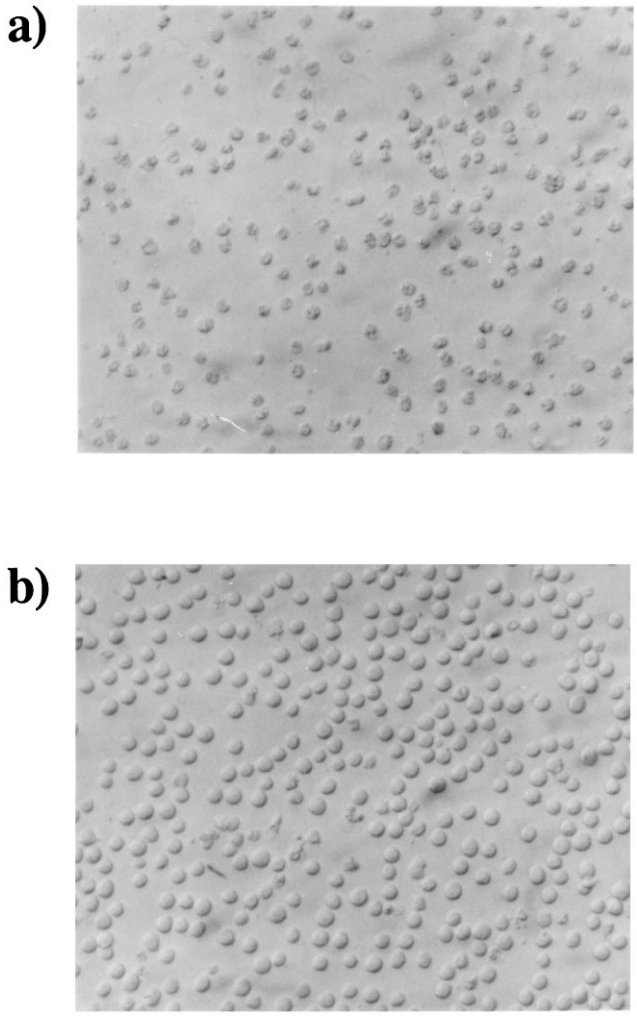


FIG. 8. Ectopic expression of c-Myc reduces apoptosis of WEHI 231 cells induced by TPCK. Stable transfectant lines of WEHI 231, prepared with either the pSV2neo vector (S10-WEHI 231) (a) or the pM21 c-*myc* expression vector (P17-WEHI 231) (b), were treated with 25 μM TPCK for 40 h. Cells were photographed under phase contrast at a magnification of ×200.

alone for 2 h or with anti-Ig alone for 1.5 h and RNA was isolated. Northern blot analysis demonstrated that TPCK blocked the five- to eightfold induction of c-*myc* RNA normally resulting from treatment with anti-Ig for 1.5 h (Fig. 3). No change in the basal level of c-*myc* RNA was noted within 0.5 h of treatment with TPCK alone, whereas a decline was observed over the subsequent 90-min period. These kinetics are consistent with the time necessary to see the drop in mRNA expression following the decline in transcription due to inhibition of NF-κB/Rel complex formation. Equal RNA loading and integrity was confirmed by rehybridization with a probe for the housekeeping gene coding for glyceraldehyde-3-phosphate dehydrogenase (Fig. 3). Thus, TPCK treatment of WEHI 231 cells suppressed basal c-*myc* expression and prevented the normal activation of c-*myc* mRNA by anti-Ig treatment.

TPCK induces apoptosis of WEHI 231 cells. Having confirmed that TPCK prevents the normal increase in c-*myc* expression upon treatment with anti-Ig and induces a reduction in basal levels of expression of this oncogene when added alone, we tested the effects of TPCK on cell death. Cells that had been pretreated with 0, 10, 25, or 50 μM TPCK for 30 min were subjected to 13 h of anti-Ig treatment. Alternatively, cultures were treated with similar doses of TPCK alone for 13.5 h. Apoptosis was measured by DNA fragmentation assay (50). Suppression of the anti-Ig-mediated induction of NF-κB/Rel activity and c-*myc* RNA levels in WEHI 231 cells due to TPCK treatment failed to prevent apoptosis (Fig. 4). In fact, pretreatment with 10 or 25 μM TPCK appeared to enhance the extent of DNA fragmentation. Furthermore, incubation of WEHI 231 cells with 10 μM TPCK alone induced as much DNA laddering as is observed normally following a similar time of anti-Ig treatment, and more extensive fragmentation was observed following treatment with 25 μM TPCK. DNA fragmentation was detectable by about 4 h following treatment with 25 μM TPCK (data not shown). Thus, induction of apoptosis in WEHI 231 cells correlates with the drop in c-*myc* RNA levels mediated by TPCK treatment.

TPCK decreases c-Myc protein levels. To confirm that TPCK treatment indeed mediated a drop in c-Myc protein levels comparable to its effects on c-*myc* mRNA expression, immunoblot analysis was performed. Nuclear extracts were prepared from WEHI 231 cells in exponential growth and after 1, 2, 4, 6, or 8 h of TPCK treatment. As seen in Fig. 5A, a drop in the level of c-Myc protein in the nucleus was observed within 2 h and levels continued to decline for up to 8 h of treatment. These findings are consistent with the kinetics of the c-*myc* RNA profiles obtained as described above and confirm that TPCK treatment causes a significant drop in c-Myc protein levels that correlates with the induction of apoptosis.

Ectopic expression of c-Myc attenuates apoptosis of WEHI 231 cells induced by TPCK. Since the drop in c-*myc* expression mediated by TPCK correlated with activation-induced cell death, we next sought to determine whether ectopic expression of c-Myc protein in WEHI 231 cells would prevent apoptosis. Stable transfectants were made with the c-*myc* expression vector pM21 (9), which is driven by the Moloney murine leukemia virus long terminal repeat (52), and clonal lines were isolated by limiting dilution. As controls, pSV2neo-transfected clones were similarly prepared. Twenty pM21-transfected clones and six pSV2neo-transfected clones were isolated. These were as-

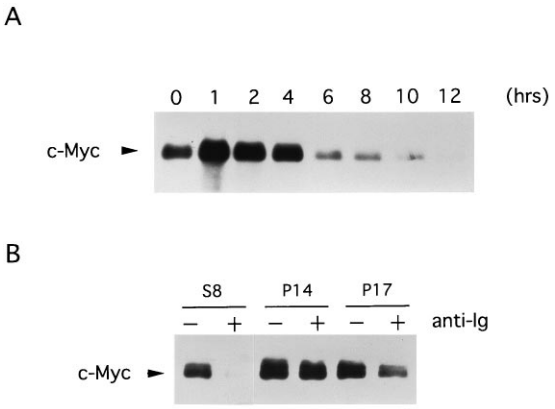


FIG. 9. Levels of c-Myc protein remain elevated following anti-Ig treatment of pM21 c-*myc*-transfected lines. (A) Anti-Ig treatment reduces expression of c-Myc protein in parental WEHI 231 cells. Nuclear proteins were extracted from WEHI 231 cells in exponential growth (0 h) or following treatment with anti-Ig for the indicated times and subjected to immunoblotting using an affinity-purified c-Myc antibody prepared against the whole murine c-Myc protein. (B) Maintenance of c-Myc expression in pM21-transfected lines. Nuclear proteins were prepared from WEHI 231 stable transfectants S8-WEHI 231 (pSV2neo) and P14- and P17-WEHI 231 (pM21 c-*myc* expression vector) in exponential growth (–) or following 14 h of treatment with anti-Ig (+) and analyzed for c-Myc protein expression as described for panel A.

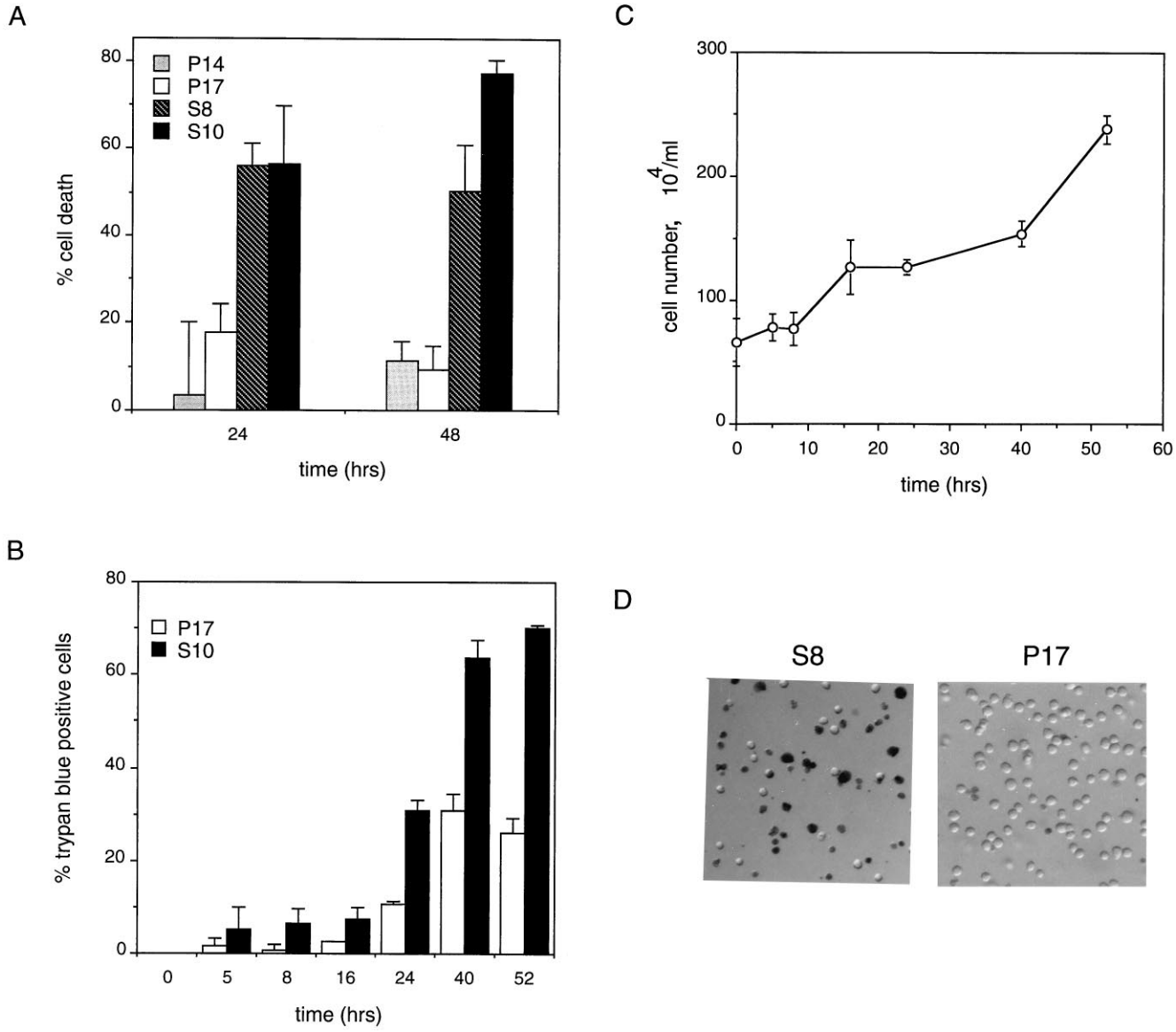


FIG. 10. Ectopic expression of c-Myc prevents apoptosis of WEHI 231 cells induced by anti-Ig. (A) MTS assay. Stable WEHI 231 clones transfected with either pSV2neo (S8- and S10-WEHI 231) or pM21 c-*myc* expression vector (P14- or P17-WEHI 231) were treated in duplicate with anti-Ig for 24 or 48 h and analyzed for cell viability by conversion of MTS dye to its formazan product. Data are plotted as percent viable cells relative to untreated control cultures. (B and C) Trypan blue exclusion assay. Cultures of clones S10-WEHI 231 (pSV2neo) and P17-WEHI 231 (pM21) were incubated in triplicate in the absence or presence of anti-Ig, and trypan blue-positive (dead) cell numbers were determined (B) or numbers of trypan blue-excluding (viable) cells for the P17-WEHI 231 line were plotted as a function of time to display the time course of changes in numbers (C). (D) Trypan blue staining. Cultures of clones S8-WEHI 231 (pSV2neo) and P17-WEHI 231 (pM21) were incubated in the absence or presence of anti-Ig for 24 h and photographed at magnification of ×100 after staining with trypan blue.

sayed by immunoblotting for c-Myc expression. The pM21 stable clones contained approximately normal levels of c-Myc protein compared with control or pSV2neo lines; however, in approximately half of the clones elevated levels of c-Myc protein were retained following TPCK treatment (Fig. 5B and data not shown). Clones retaining c-Myc expression were tested for the extent of apoptosis following TPCK treatment by using four criteria: fluorescence-activated cell sorter (FACS) analysis of PI staining, conversion of MTS to its formazan product, trypan blue exclusion, and visual appearance. For FACS analysis, stable transfectant WEHI 231 lines were treated with 25 μM TPCK for 5 h. Cells were harvested and analyzed by flow cytometry for the percentage of cells expressing sub-G_0/G_1 levels of DNA (i.e., apoptotic cells). Two clones transfected with control pSV2neo (S10- and S12-WEHI 231) exhibited significant levels of apoptosis (34% ± 3% when results obtained with each clone were averaged). In contrast, nine clones (i.e., the pM21 clones P2-, P4, P8-, P14-, P17-, P24-, P25-, P35-, and P36-WEHI 231) stably expressing c-Myc were markedly less sensitive to TPCK, with only 12% ± 3% of the cells affected by the treatment. pSV2neo- and pM21 expression vector-transfected cells not treated with TPCK showed 2 and 3% apoptosis, respectively. A more complete time course of the effects of TPCK on cell viability was performed. Two of the lines expressing c-Myc, i.e., P14- and P17-WEHI 231, and two pSV2neo-transfected lines (S8- and S10-WEHI 231) were treated in triplicate with 25 μM TPCK, and cell viability was monitored by the MTS dye conversion assay

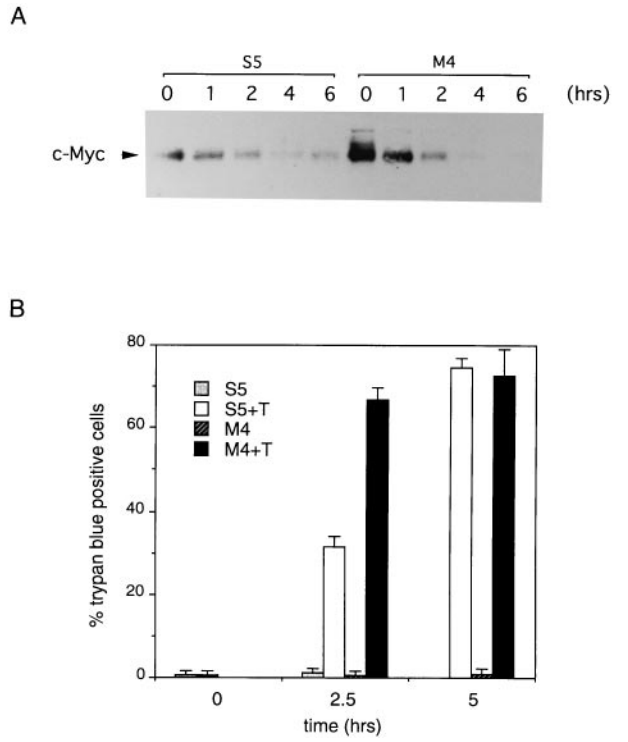
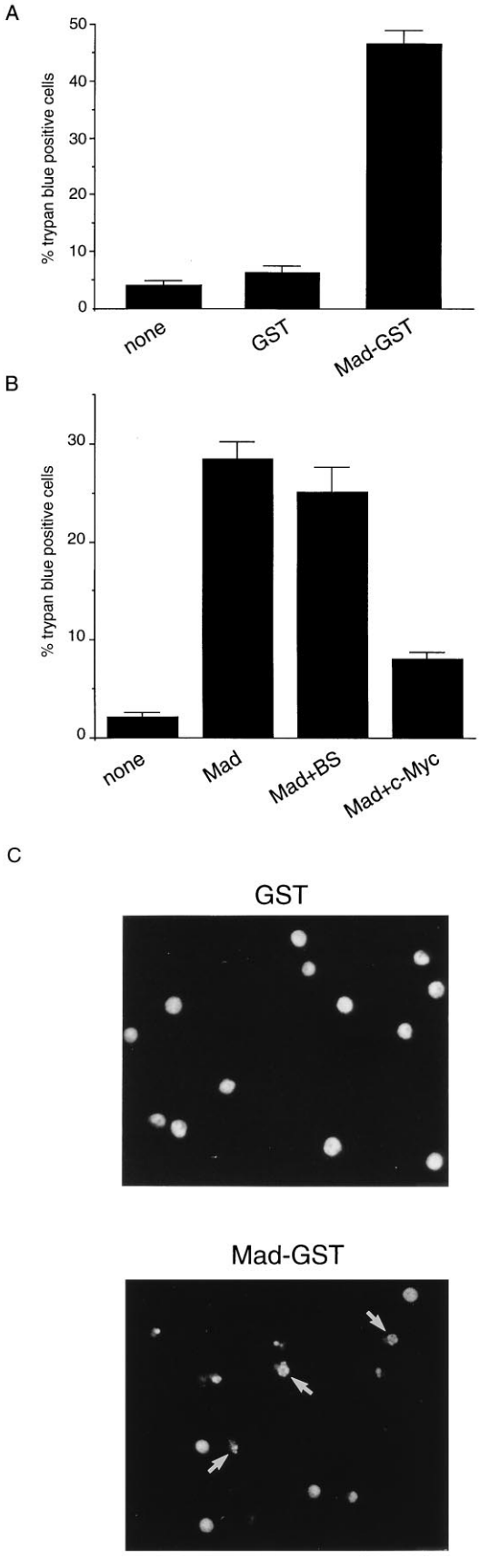


FIG. 11. TPCK treatment of WEHI 231 stable lines expressing c-*myc* under the control of the CMV promoter causes a large, rapid drop in c-Myc expression and accelerated induction of apoptosis. WEHI 231 clones S5-WEHI 231 and M4-WEHI 231, stably transfected with pSV2neo alone and pRc-CMV-myc plus pSV2neo, respectively, were incubated in the absence or presence of 25 μM TPCK for the indicated periods. (A) Nuclear proteins (40 μg per sample) were isolated and subjected to immunoblotting using an affinity-purified c-Myc antibody. (B) Apoptosis. WEHI 231 clones S5-WEHI 231 and M4-WEHI 231 were treated in triplicate with 25 μM TPCK, and cell death was assessed after 2.5 and 5.0 h by trypan blue assay, as described in the legend to Fig. 7B.

(Fig. 6). Extensive cell survival was noted even after 36 h of treatment of the Myc-expressing lines. In contrast, only 20% of the two pSV2neo-transfected cell lines were viable over a similar time frame. To further quantitate the effects of c-Myc on survival, the P17-WEHI 231 and the S10-WEHI 231 lines were treated in triplicate with 25 μM TPCK and assessed with trypan blue for live (excluding) versus dead (positive) cells. As seen in Fig. 7A, TPCK treatment resulted in extensive loss of viable cells for the SV2neo control line within 24 h. As expected, little cell death was noted in the absence of TPCK treatment (Fig. 7B). In contrast, treatment of the c-Myc-expressing line led to an initial period of cell death, which reached maximally to approximately 40% of the cells. The surviving cells continued to proliferate, and growth continued with a normal doubling time. Consistent with this finding, TPCK treatment of cultures of pSV2neo clones resulted in the emergence of cells with the morphologic appearance of apoptotic cells, whereas extensive survival of cells was noted upon similar treatment of cultures of c-*myc*-transfected lines. For example, Fig. 8 illustrates a comparison of the effects of TPCK on S10-WEHI 231 (panel a) and on P17-WEHI 231 (panel b). Thus, the c-*myc*-transfected culture displayed extensive survival following the TPCK treatment and proliferation was noted even after 72 h of TPCK treatment of the c-Myc-expressing P17-WEHI 231 line (data not shown). Taken together, these results demonstrate that TPCK-induced apoptosis is significantly reduced by constitutive expression of c-Myc protein in WEHI 231.

Ectopic expression of c-Myc attenuates anti-Ig-mediated apoptosis of WEHI 231 cells. We next assessed whether ectopic c-Myc expression protects against anti-Ig receptor-mediated cell killing. Two pSV2neo control lines and two c-Myc-expressing lines were treated with anti-Ig. To verify the expression of c-Myc protein in the lines following anti-Ig treatment, the levels of c-Myc in pSV2neo S8-WEHI 231 cells and the P14- and P17-WEHI 231 lines, as well as the levels in anti-Ig-treated parental cells, were compared (Fig. 9). As expected, anti-Ig treatment of parental WEHI 231 cells results in an initial increase in c-Myc protein levels followed by a decline (Fig. 9A), consistent with the changes in the levels of c-*myc* RNA (35, 40). In the two c-*myc*-transfected lines, extensive expression of c-Myc protein was noted even after 14 h, whereas the S8-WEHI 231 line displayed a drop similar to that in the parental line (Fig. 9B). To examine the time course of the effects of anti-Ig, cell death was monitored over a 48-h period by using conversion of MTS to its formazan product (Fig. 10A). The two pSV2neo-transfected lines displayed extensive loss of cell viability by 24 h of anti-Ig treatment. The time course of the effects of anti-Ig on cell death is consistent with previous findings (10, 14, 27). In contrast, significantly less cell death was observed following anti-Ig treatment of two *myc*-transfected lines, with approximately 90% viable cells remaining after 48 h of treatment of the P14- and P17-WEHI 231 lines (Fig. 10A). Trypan blue analysis of similarly treated cell cultures, carried out over a 52-h time frame, demonstrated greater than 70% loss of cell viability for the pSV2neo (S10-WEHI 231) line compared with less than 30% for the P17-WEHI 231 c-*myc*-transfected line (Fig. 10B). Furthermore, proliferation of these cells was clearly evident after 52 h of anti-Ig treatment (Fig. 10C). In Fig. 10D, a representative comparison of trypan blue-stained cultures of control (S8-WEHI 231) and c-*myc*-expressing (P17-WEHI 231) cells treated with anti-Ig for 24 h, depicting the effects of c-Myc expression on cell survival, is given. Thus, ectopic expression of c-Myc affords WEHI 231 cells substantial protection from anti-Ig-mediated apoptosis.

TPCK treatment of CMV promoter-*myc* expression vector stable WEHI 231 transfectants results in a dramatic decline in c-Myc levels and more rapid induction of apoptosis. Stable transfectants of WEHI 231 cells were also prepared by using the pRc-CMV-myc expression vector. In this construct, Myc 2 protein, the predominant form initiated at the AUG codon start site, is expressed under the control of the cytomegalovirus (CMV) promoter. A total of 20 pRc-CMV-myc-expressing clones were selected with the antibiotic G418. In addition, five clones containing the pSV2neo parental vector were similarly isolated. Cells were incubated in the presence or absence of 25 μM TPCK and analyzed for expression of c-Myc and for induction of apoptosis. Expression of pRc-CMV-myc led to enhanced expression of c-Myc protein as indicated by Western blotting (Fig. 11A and data not shown). The level of c-Myc protein was dramatically inhibited by TPCK treatment. The extent of the drop was more complete than that in control cells. This result is consistent with the notion that the CMV promoter, which contains five full and six partial NF-κB elements (16), is driven predominantly by Rel factor binding in WEHI 231 cells, and upon inhibition of NF-κB/Rel binding by TPCK treatment the level of activity of this promoter dropped dramatically. When the pRc-CMV-myc clones of these cells were subjected to analysis for apoptosis, the induction of cell death following TPCK treatment appeared accelerated. For example, Fig. 11B shows a typical comparison for clones transfected with pRc-CMV-myc (M4-WEHI 231) and clones transfected with the pSV2neo control (S5-WEHI 231) using trypan blue exclu-



sion. More extensive cell death was noted within 2.5 h in the CMV-myc-transfected line than in control cells. Similar results were seen by DNA ladder formation (data not shown), although this method is less quantitative. These results suggest that the more precipitous decline of the elevated initial levels of c-Myc protein in the lines transfected with pRc-CMV-myc resulted in a more dramatic induction of apoptosis.

Microinjection of Mad1 induces apoptosis. The data presented above indicated that expression of c-*myc* can curtail apoptosis induced by TPCK or anti-Ig. To determine whether down-regulation of c-*myc* is sufficient to induce apoptosis of WEHI 231 cells, we microinjected agents known to specifically counteract Myc function. The effects of introduction of Mad1, a specific binding partner of Max (8), were monitored. Max, which potentiates the binding activity of Myc-Max heterodimers through its basic region, has been shown to play a central role in functional activity of c-Myc (3, 8). Introduced Mad1 has been found to compete with c-Myc for interaction with Max and thus to serve as a competitive inhibitor of functional c-Myc (7). Microinjection of Mad1-GST protein significantly increased death of WEHI 231 cells, as judged by trypan blue positive staining (Fig. 12A and Table 1). In contrast, microinjection of control GST protein had little effect on induction of cell death. Apoptosis of these cells was confirmed by the appearance of condensed chromatin following microinjection of Mad1-GST (Fig. 12C, bottom panel) compared with microinjection of GST alone (Fig. 12C, upper panel), as visualized by PI staining 4.5 h after microinjection. In order to verify the specificity of this effect, we microinjected a Mad1 expression vector in the presence or absence of the Myc expression vector pM21. Microinjection of Mad1 expression vector similarly induced cell death, indicating that apoptosis was not due to general toxicity of the protein preparation (Fig. 12B). The level of cell death was reduced significantly upon comicroinjection of a c-*myc* expression vector but not of control Bluescript vector DNA. These results indicate that the down-regulation of functional c-Myc is sufficient to induce apoptosis of WEHI 231 cells.

DISCUSSION

The results presented here demonstrate a central role for the drop in c-*myc* expression in the induction of apoptosis in WEHI 231 B cells, a model for B-cell tolerance induction via clonal deletion (41). Treatment of WEHI 231 cells with the protease inhibitor TPCK, which reduced NF-κB/Rel activity, caused decreased c-*myc* mRNA and protein levels. Extensive apoptosis resulted from the TPCK treatment. Ectopic c-Myc expression led to enhanced escape of WEHI 231 B cells from the apoptosis induced by treatment with either TPCK or anti-Ig, suggesting that the decreased expression of c-Myc played a direct role in the activation of the physiologic cell death program. Furthermore, when a CMV promoter–c-*myc* expression

FIG. 12. Expression of the Max binding partner Mad1 induces apoptosis of WEHI 231 cells. (A) Microinjection of Mad1-GST protein. WEHI 231 cells, unmicroinjected (none) or microinjected in duplicate with 1 mg of GST or Mad1-GST per ml, were analyzed for cell death via trypan blue staining. Cell numbers are given in Table 1. (B) Microinjection of Mad1 expression vector. WEHI 231 cells were unmicroinjected (none) or microinjected in duplicate with an expression vector for Mad1 alone or in combination with empty Bluescript DNA (BS) or the c-*myc* expression vector PM21 (c-Myc). Cell death was monitored via trypan blue exclusion, as described in Materials and Methods. (C) PI staining. WEHI 231 cells were microinjected with GST (upper panel) or Mad1-GST protein (lower panel). After 4.5 h, cells were stained with PI and photographed at a magnification of ×200 with TMAX film. Arrows point to nuclei of cells with extensive DNA condensation typical of apoptosis.

TABLE 1. Microinjection of WEHI 231 cells with Mad protein or expression plasmid induces cell death[a]

Microinjected solution	No. of viable cells/ total (duplicates)	% Nonviable cells (mean ± SEM)
Expt 1 (protein)		
None	127/131, 116/122	4.0 ± 0.9
GST (1 mg/ml)	121/131, 136/143	6.2 ± 1.3
Mad-GST (1 mg/ml)	83/148, 79/155	46.5 ± 2.5
Expt 2 (expression plasmid)		
None	109/112, 121/123	2.1 ± 0.5
Mad	131/179, 124/178	28.5 ± 1.7
Mad + BS[b]	91/126, 107/138	25.1 ± 2.6
Mad + Myc	140/151, 135/148	8.0 ± 0.7

[a] WEHI 231 cells were microinjected with the indicated solutions of proteins (experiment 1) or expression vectors (experiment 2). After 20 h, viable cell counts were determined via trypan blue exclusion.
[b] BS, empty Bluescript DNA.

vector was used, the enhanced drop in c-Myc expression was found to correlate with a more rapid induction of apoptosis. To confirm this role, we employed microinjection analysis. Microinjection of either Mad1 protein or a vector expressing Mad1 induced apoptosis of WEHI 231 cells directly. Consistent with these conclusions, previous results had indicated a correlation between the drop in c-*myc* levels and the induction of apoptosis in WEHI 231 and other B-cell lines at a similar stage of development, i.e., CH31 or CH33. For example, treatments, such as anti-Ig and TGF-β1 (10, 27, 36, 40, 56), that result in decreased c-*myc* expression lead to the induction of apoptosis. In contrast, agents that induce c-*myc* expression but fail to cause the subsequent drop, including anti-IgD and lipopolysaccharide, all fail to induce apoptosis (14, 29, 54, 56). Furthermore, addition of an antisense c-*myc* oligonucleotide that surprisingly stabilized c-Myc protein expression in WEHI 231 and CH31 cells prevented apoptosis induced by anti-Ig or TGF-β1 treatment (23; also data not shown). We have recently found that the T-cell CD40 ligand, which protects against anti-IgM-induced apoptosis of WEHI 231 cells and leads to continued cell growth and viability (55), causes maintenance of NF-κB and c-*myc* expression (47). Our recent results also show that ectopic c-*myc* expression protects against TGF-β1-mediated apoptosis of WEHI 231 cells (5). Taking these results together with the findings presented here, we conclude that the drop in functional Myc leads to physiologic death of WEHI 231 B cells.

In contrast to these results, evidence indicating that overexpression of c-*myc* promotes apoptosis, in particular following loss of a growth promoting signal, has accumulated. Thus, growth factor deprivation of fibroblasts, myeloid cells, or smooth muscle cells overexpressing c-Myc, via either an estrogen-inducible estrogen receptor fusion protein or an ectopic c-*myc* expression vector, induced accelerated apoptosis (6, 11, 22). Furthermore, interference with T-cell receptor-mediated upregulation of c-*myc* by the use of c-*myc* antisense oligonucleotides ablated apoptosis (48). Similarly, antisense oligonucleotides against c-*myc* rendered fibrosarcoma cells resistant to tumor necrosis factor alpha mediated cell death (31). These findings support a role for c-*myc* in promoting apoptosis, although the exact mechanism for c-Myc action in these diverse systems is unclear. In fibroblasts, the interaction of c-Myc with Max appears obligatory to promote the induction of apoptosis (3). In the WEHI 231 system, overexpression of Mad1, which competes for Max binding and negatively regulates c-Myc (7, 8), induced apoptosis. These findings demonstrate a direct role for Mad1, and therefore Max, in apoptosis of B cells, although in this case the reduction in the level of c-Myc activity correlates with induction of cell death. Interestingly, we have recently found that TPCK and TGF-β1 treatments of WEHI 231 cells lead to activation of Mad1 expression that precedes apoptosis, suggesting that this protein plays a role in B-cell death under physiologic circumstances (58).

Germinal centers are active sites of normal B-lymphocyte differentiation; hypermutation and proliferation of activated B cells occurs in these microenvironments following antigen immunization (18, 32). Ig gene mutation can also lead to formation of self-reactive antigens. It has been suggested that these B lymphocytes are eliminated within the germinal centers, and recent evidence indicates that soluble antigen causes enhanced apoptosis of germinal-center B cells (44, 49). Interestingly, CD40 ligand has been found to override apoptosis in germinal centers, raising the intriguing possibility that NF-κB/Rel and c-Myc proteins play similar roles in apoptosis within the germinal center.

$Bcl\text{-}X_L$ expression can result in survival of WEHI 231 from anti-Ig-induced physiologic cell death (24). Surprisingly, recent evidence has indicated that in this case $Bcl\text{-}X_L$ fails to prevent arrest of cells in the G_1/S phase (41). These findings suggest that anti-Ig treatment induces a change in signalling apart from those responsible for the arrest of the cell cycle that induces apoptosis of WEHI 231 cells and that $Bcl\text{-}X_L$ compensates for this alteration, thereby overriding induction of apoptosis of this line. Consistent with these observations, $Bcl\text{-}X_L$ also ablates TPCK-mediated induction of apoptosis of WEHI 231 cells but fails to prevent the drop in c-Myc protein expression (data not shown).

The serine proteinase inhibitor TPCK has been demonstrated to be a potent, specific inhibitor of NF-κB/Rel activity (28). It acts via stabilization of the normally labile IκBα protein, preventing its decay. For example, in the presence of TPCK the short half-life of IκBα protein in WEHI 231 cells (20 min) is significantly lengthened (to >1.5 h) (42; also data not shown). The ability to modulate IκBα and thus NF-κB is specific to TPCK. Other similar proteinases do not have such an activity. Thus, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride have not been found to affect NF-κB binding, and these inhibitors failed to induce apoptosis of WEHI 231 cells (data not shown). In addition, we similarly tested the effects of TPCK treatment on NIH 3T3 fibroblasts. NIH 3T3 cells do not appear to express significant levels of NF-κB/Rel (34). Treatment of these cells with 25 μM TPCK only slightly affected their growth, while higher doses, e.g., 50 to 100 μM TPCK, caused arrest of cell growth but not apoptosis (59). These findings suggest that it is the effects of TPCK on NF-κB/Rel and thus on c-*myc* that lead to the apoptosis of these B lymphocytes (59). The observation that anti-Ig treatment of WEHI 231 cells results in a rapid decline in NF-κB/Rel binding (35), and thus in c-*myc* expression, rather than activation as has been found for splenic B lymphocytes (15, 37) is likely the result of developmental control that is also responsible for the induction of apoptosis of these immature B cells. These findings, therefore, implicate not only NF-κB/Rel factors, as has been found by other groups (1, 57), but also members of the IκB family of inhibitory proteins that regulate their expression as potential early mediators of signals controlling apoptosis in these B cells.

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